THE ROLE OF *MYCOPLASMA SYNOVIAE* IN COMMERCIAL LAYER *E.COLI* PERITONITIS SYNDROME AND *MYCOPLASMA GALLISEPTICUM* INTRASPECIFIC DIFFERENTIATION METHODS

by

ZIV RAVIV

(Under the Direction of Stanley H. Kleven and Zhen Fu)

ABSTRACT

Mycoplasmas are bacteria that belong to the class *Mollicutes*. Mycoplasmas are found in humans and animals, and the species that were recognized as pathogens of domestic poultry include *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in chickens and turkeys, and *Mycoplasma meleagridis* and *Mycoplasma iowae* in turkeys.

MS is an important pathogen of domestic poultry, and is prevalent in commercial layers. During the last decade *Escherichia coli* (*E. coli*) peritonitis became a major cause of layer mortality. Commercial layers at the onset of lay were challenged through the respiratory tract with a virulent MS strain or a virulent avian *E. coli* strain, or both. A significant *E. coli* peritonitis mortality was detected in the MS plus *E. coli* challenged group, and severe tracheal lesions and moderate body cavity lesions were detected only in the MS challenged groups. The results demonstrate a possible pathogenesis mechanism of respiratory origin to the layer *E. coli* peritonitis syndrome, show the MS pathological
effect in layers, and suggest that a virulent MS strain can act as a complicating factor in the layer \textit{E. coli} peritonitis syndrome.

MG is the most pathogenic and economically significant mycoplasma pathogen of poultry. It has become increasingly important to differentiate between MG strains and isolates. We designed a specific and sensitive polymerase chain reaction (PCR) for the amplification of the complete MG intergenic spacer region (IGSR) segment. The MG IGSR sequence was found to be highly variable (discrimination ($D$) index of 0.950) among a variety of MG laboratory strains, vaccine strains, and field isolates. The sequencing of the MG IGSR appears to be a valuable single-locus sequence typing (SLST) tool for MG isolate differentiation in diagnostic cases and epizootiological studies.

MG control with live vaccines was demonstrated to efficacious, and the development of live vaccines usually requires the involvement of several vaccine and challenge strains. We developed real-time PCR assays that absolutely differentiated between the five commercial and laboratory vaccine strains: F, ts-11, 6/85, K5831, K5054, and the common challenge strain R$_{low}$. The assay was also tested \textit{in vivo} and successfully differentiated between the vaccine and the challenge strains in a quantitative manner.

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PERITONITIS SYNDROME AND *Mycoplasma gallisepticum* INTRASPECIFIC

DIFFERENTIATION METHODS

by

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DEDICATION

“I remember the devotion of your youth, how you loved me as a bride, following me in the desert, in a land unsown”. (Jeremiah 2.2).

This dissertation is dedicated to my beloved wife Ronit, who joined me on this unusual journey and unconditionally supported me along the way.
ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge my major professor Dr. Stanley H. Kleven, who encouraged me to take this journey in the middle of my career, was there for me when things did not go according to the plan, and led me in his unique way to places that I would never reach without him. Sadly, I acknowledge the enormous contribution and dedication to my program of my co-major professor Dr. Barry B. Harmon who passed away in January 2007. A special acknowledgement to our department head Dr. John Glisson, for supporting my program and for making it all possible. I would like to thank the members of my committee Dr. Zhen Fu, Dr. Lillian Jaso-Friedmann and Dr. Mark W. Jackwood for their guidance. Without the help of the staff and students at the Poultry Diagnostic and Research Center this would have been a near impossible task. I would like to thank especially my labmates Dr. Naola Ferguson, Mrs. Victoria Laibinis, and Mrs. Ruth Wooten for looking after me during these years.
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6 DISCUSSION AND CONCLUSIONS...............................................................150
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Poultry production has changed dramatically during the last half century. It has turned industrialized and integrated with high throughput and continues production complexes; in other words, large multiage operations in confined geographical areas. This cost driven trend made the poultry industry highly efficient but also vulnerable to some infectious disease hazards. One such group of disease agents are the avian mycoplasmas, due to the chronic disease and the life long infection that they cause. Avian mycoplasmas, predominantly *Mycoplasma gallisepticum* (MG), became to be one of the costliest diseases problems to commercial poultry production. The currently available measures for the control of avian mycoplasmas offer only a partial relief, and the search for better diagnostics, infection source tracing and prophylactic methods for avian mycoplasmas continue.

This research focused on current problems in the area of avian mycoplasmology from the perspective of the Mycoplasma Laboratory in the Poultry Diagnostic and Research Center, the University of Georgia. The research was composed of 3 different studies. The first study of this research focused on the role of endemic *Mycoplasma synoviae* (MS) infection in US commercial layer complexes, and whether MS challenge at the onset of lay could be associated with the increase in *E. coli* peritonitis mortality reported during the last decade. The two other studies focused on the development of MG intraspecific differentiation methods for infection tracking and live vaccine evaluation purposes.
*Mycoplasma synoviae* is an important pathogen of domestic poultry, causing economic losses to the poultry industry (16). The role of MS in egg production of commercial layers is questionable. Previous studies have revealed that MS infection is rather common in commercial layers (6, 10, 26). In the US table egg industry, most pullets are MS free in the rearing phase but become infected with MS after their introduction into the multiage laying complexes. The economic impact of MS infection in layers is usually estimated to be low because little or no effect either on egg production (2, 27) or egg quality has been reported (2, 23). During the last decade *Escherichia coli* (*E. coli*) peritonitis has been considered to be a major cause of layer mortality in the US and other countries (Kenton Kreager, personal communication, 33). Layer *E. coli* peritonitis is characterized by acute mortality without prior morbidity or significant impact on egg production. Peritonitis with yolk material deposited in the body cavity and polyserositis are the two main necropsy observations associated with the disease. The occurrence of *E. coli* peritonitis in layer flocks appears to have two mortality peaks along the laying cycle. The first increase in mortality occurs around the peaking period in egg production, and suspected to be of respiratory origin. The second increase in mortality is observed during the late lay period at the age of fifty weeks and onward, and suspected to be of vent origin due to vent trauma (Kenton Kreager, personal communication). The increase in mortality due to the *E. coli* peritonitis which occurs during the peaking period varies between few tenths of percent to over one percent per week in severe cases (33). Field impressions suggested that some layer flocks suffer from the peaking period *E. coli* peritonitis mortality simultaneously with the point in time when they turn serologically positive for MS. Scientific reports on layer *E. coli* peritonitis are scarce, but in a flock survey conducted by Vandekerchove *et al.* no association could be found between outbreaks of *E. coli* peritonitis mortality and infection with MG, MS or other avian respiratory
pathogens (34). This study addressed the query whether MS challenge at the onset of lay could be associated with the reported *E. coli* peritonitis mortality during the peaking period. The study results demonstrated a possible pathogenesis mechanism of respiratory origin to the layer *E. coli* peritonitis syndrome, showed the MS pathological effect in layers, and suggested that a virulent MS strain can act as a complicating factor in the layer *E. coli* peritonitis syndrome.

MG is the most pathogenic and economically significant mycoplasma pathogen of poultry. Economic losses from condemnation or downgrading of carcasses, reduced feed and egg production efficiency, and increased medication costs are additional factors that make this one of the costliest disease problems confronting commercial poultry production worldwide (21). It has become increasingly important to develop methods to characterize and identify MG strains and strain variability. Reliable methods for the differentiation of MG strains play a pivotal role in understanding the epizootiology and spread of the disease because they generate the information necessary to identify and track new outbreaks. Also, the increased use of live MG vaccines requires more powerful tools to trace the source of infection and to differentiate vaccine strains from circulating field isolates. Recognition of intraspecific (strain) genotypic and phenotypic heterogeneity may be done by serologic methods (18, 30) or electrophoretic analysis of cell proteins (15). However, molecular techniques can be more sensitive and discriminatory for the differentiation of MG strains (22). Sequencing methods have been introduced as a new approach for studying the molecular epidemiology of bacterial pathogens (5). Multilocus sequence typing (MLST) of housekeeping genes has been demonstrated to be a highly transferable typing method, readily applicable to a wide variety of bacteria, which has contributed to the understanding of global epidemiology and population structure of infectious diseases (24). Also the sequencing of a single variable chromosomal locus (single-locus
sequence typing (SLST)) was demonstrated as a promising approach for the detection of bacterial strain variation (19). It was demonstrated recently that gene-targeted sequencing (GTS) analysis of MG surface-protein genes is a reproducible typing method with satisfactory discriminatory power to separate isolates from unrelated outbreaks, and to identify closely related isolates (7). Unlike other widely used MG typing methods the sequencing methods do not require the isolation of the tested organism in a pure culture. Mixed infection with MG and saprophytic mycoplasmas is a common occurrence in poultry flocks (25), and the independence of the sequencing methods on the recovery of MG in a pure culture is a significant advantage.

Some of the genes coding for rRNA molecules of the genus *Mycoplasma* are organized in operons and arranged in the order 5’ 16S-23S-5S 3’ in which the individual rRNA genes are separated by internal transcribed spacer regions. The intergenic spacer region (IGSR) between the 16S and 23S rRNA genes of mycoplasmas has been shown to lack tRNA genes and to be variable in sequence and length among mycoplasma species (32). It has been used as a genetic marker for comparing phylogenetic relationships of genetically closely related species among not only the mycoplasmas (12, 31), but also other bacterial species (9, 11, 13, 20). MG contains two sets of rRNA genes (5S, 16S, and 23S) in its genome, but only one of the two is organized in an operon cluster and contains a unique 660 nucleotide IGSR between the 16S and the 23S rRNA genes (28). This study objective was to determine the level of the intraspecific genotypic polymorphism of the MG 16S-23S rRNA IGSR, and to evaluate the discriminatory power of this single chromosomal locus for MG isolates. Sequencing of the MG IGSR appears to be a valuable single-locus sequence typing (SLST) tool for MG isolate differentiation in diagnostic cases and epizootiological studies.
MG traditional prevention and control programs were based on strict biosecurity, surveillance (serology, culture, and molecular identification), and eradication of infected breeder flocks. The rapid expansion of poultry production in restricted geographical areas and the consequent recurring MG outbreaks necessitated the implementation of additional measurements. Vaccination with live MG vaccines has become an accepted management tool for the control of MG in chickens and in some situations it is the preferred control option, particularly when multiple age flocks are housed on the same site (3). Currently there are 3 commercially licensed vaccines, containing living cultures of either F (1), 6/85 (4) or ts-11 (35) strains of MG. Live vaccine development and evaluation require studies that involve two or more MG strains in the same experimental setup. Protection study formats can include only one vaccine strain and one challenge strain (29), or a few vaccine strains in different experimental groups challenged by the same virulent strain (8). Displacement studies, to evaluate the capability of vaccine strains to displace a virulent strain, utilize several vaccine strains and a challenge strain (17). The study of the immune mechanisms by which the MG vaccines conferred protection from challenge also requires the involvement of at least two strains (14). In all the different MG live-vaccine evaluation study formats the involved strains could not be well differentiated and analyzed separately from one another once they were introduced into the experimental system. This lack of ability to differentiate between the participating strains limits the level of control and the amount of information that could be gained from MG vaccine evaluation studies. This study objective was to develop a research tool to allow the qualitative and quantitative differentiation between MG strains utilized in vaccine evaluation studies and to improve the reliability and efficiency of these studies. A real-time PCR with a dual-labeled probe (TaqMan) has several advantages for microorganisms strain differentiation: the superior
sensitivity (detection of 1 to 10 copies per 5 µl sample); improved specificity endowed by 3 hybridizing oligo nucleotides (two primers and a probe); the inherent quantitative nature of the reaction; the possibility of detecting more then one reaction product by using several fluorescent dyes (multiplex reaction); and the convenience of a one step reaction. This study present the concept of quantitative strain differentiating real-time PCR for the differentiation between MG strains in live vaccine evaluation studies.

References


CHAPTER 2
LITERATURE REVIEW

The Class *Mollicutes*

Mycoplasmas belong to the class *Mollicutes* which is derived from the Latin ‘*mollis*’ meaning ‘soft’ and ‘*cutis*’ meaning ‘skin’ and this name refers to the fact that these organisms lack a conventional bacterial cell wall and are surrounded only by a thin membrane. Mollicutes are the smallest and simplest known free-living and self-replicating forms of life. They are bacteria of gram-positive origin, as indicated by their 16S rDNA. But rather than being primitive, they diverged about 600 million years ago, by regressive evolution and genome reduction, from more complex ancestors on the branch of the bacterial phylogenetic tree containing the lactobacilli, bacilli, clostridia and streptococci (174).

According to the current taxonomy of prokaryotes, the mollicutes belong to the phylum *Firmicutes* (46). This phylum is reserved for gram-positive bacteria with low G+C content of the genome and it also comprises the classes *Bacilli* and *Clostridia*. The phylum *Firmicutes* belong to the domain *Bacteria*. The domain concept, which was introduced by Woese and co-workers (190), is based on evolutionary trees derived from 16S (or 16S-like) rDNA sequence analysis, and this concept has now also been accepted in the 2nd edition of Bergey’s Manual (46). All prokaryotic organisms are now arranged within one of the two domains *Archaea* or *Bacteria* (22).
The members of the class *Mollicutes* are characterized by their small genome size (0.58 – 2.2 Mbp), a low G+C content (23 – 40 mol%) of the genome and a permanent lack of cell wall. The latter is account for the “fried egg” colony morphology of non-helical and non-motile mollicutes, and the resistant to antibiotics affecting cell wall synthesis (e.g., penicillin). The properties distinguishing mollicutes from other bacteria are summarized in Table 2.1 (164). The valid description of the members of the class *Mollicutes* needs to follow the rules of the Minimum Standard Document (65), the first version of which was published in 1972. The current classification of the class *Mollicutes* and the properties distinguishing the currently established taxa are presented in Table 2.2 (67).

The first extensive phylogenetic analysis based on the 16S rDNA of the mollicutes was done by Woese and co-workers (181), and in 1993 a revised taxonomy was introduced for the mollicutes (176). This classification was based on polyphasic (phylogenetic and phenotypic) approach and in addition to the six previously described genera, two new genera (*Entomoplasma* and *Mesoplasma*) were introduced (Table 2.2). Since the late 1980s tremendous weight has been given to 16S rDNA sequences in the phylogeny, taxonomy and species identification of mollicutes (23, 60, 111, 157, 158, 159, 160, 180).

Sequence data of the 16S rRNA molecule or its genes have proved to be very useful in studying the phylogeny of bacteria (150, 191). All self replicating organisms have ribosomes and rRNA and, therefore, it is possible to use 16S rRNA sequence data to construct universal phylogenetic trees in which any species may be included. The function of the 16S rRNA molecule is essential in the translation machinery of the cell and it has remained unchanged during evolution rendering this molecule a useful phylogenetic tool. A great span in phylogenetic
Table 2.1. Properties distinguishing mollicutes from other bacteria

<table>
<thead>
<tr>
<th>Property</th>
<th>Mollicutes</th>
<th>Other bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>Cholesterol present in most species</td>
<td>Cholesterol absent</td>
</tr>
<tr>
<td>Genome size</td>
<td>580–2,220 kb</td>
<td>1,050 – 10,000 kb</td>
</tr>
<tr>
<td>G+C content of genome</td>
<td>23–40 mol%</td>
<td>25–75 mol%</td>
</tr>
<tr>
<td>No. of rRNA operons</td>
<td>1 or 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1–10</td>
</tr>
<tr>
<td>5S rRNA length</td>
<td>104–113 nt&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt; 114 nt</td>
</tr>
<tr>
<td>No. of tRNA genes</td>
<td>30 (&lt;i&gt;M. capricolum&lt;/i&gt;), 33 (&lt;i&gt;M. pneumoniae&lt;/i&gt;)</td>
<td>84 (&lt;i&gt;B. subtilis&lt;/i&gt;), 86 (&lt;i&gt;E. coli&lt;/i&gt;)</td>
</tr>
<tr>
<td>UGA codon usage</td>
<td>Tryptophan codon in &lt;i&gt;Mycoplasma&lt;/i&gt;, &lt;i&gt;Ureaplasma&lt;/i&gt;, &lt;i&gt;Spiroplasma&lt;/i&gt;, &lt;i&gt;Mesoplasma&lt;/i&gt;</td>
<td>Stop codon</td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>Rifampin resistant</td>
<td>Rifampin sensitive</td>
</tr>
</tbody>
</table>

<sup>a</sup> Three rRNA operons in <i>Mesoplasma lactucae</i>.

<sup>b</sup> nt, nucleotides.
**Table 2.2.** Taxonomy and characteristics of genera of the class *Mollicutes.*

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>No. of Taxa</th>
<th>Chol. Req.</th>
<th>Phenotypic Characteristics</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycoplasmatales</em></td>
<td><em>Mycoplasmataceae</em></td>
<td><em>Mycoplasma</em></td>
<td>165</td>
<td>Yes</td>
<td>Opt. growth: 37°C</td>
<td>Humans, animals</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ureaplasma</em></td>
<td>9</td>
<td>Yes</td>
<td>Urease positive</td>
<td>Humans, animals</td>
</tr>
<tr>
<td><em>Entomoplasmatales</em></td>
<td><em>Entomoplasmataceae</em></td>
<td><em>Entomoplasma</em></td>
<td>6</td>
<td>Yes</td>
<td>Opt. growth: 30°C</td>
<td>Insects, plants</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Mesoplasma</em></td>
<td>13</td>
<td>No</td>
<td>Opt. growth: 30°C</td>
<td>Insects, plants</td>
</tr>
<tr>
<td></td>
<td><em>Spiroplasmataceae</em></td>
<td><em>Spiroplasma</em></td>
<td>85</td>
<td>Yes</td>
<td>Helical morphology</td>
<td>Insects, plants</td>
</tr>
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<td><em>Acholeplasmatales</em></td>
<td><em>Acholeplasmataceae</em></td>
<td><em>Acholeplasma</em></td>
<td>18</td>
<td>No</td>
<td>Opt. growth: 30-7°C</td>
<td>Animals, plants surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Phytoplasma</em></td>
<td>429[^b]</td>
<td>ND</td>
<td>Uncultured in-vitro</td>
<td>Insects, plants</td>
</tr>
<tr>
<td><em>Anaeroplasmatales</em></td>
<td><em>Anaeroplasmataceae</em></td>
<td><em>Anaeroplasma</em></td>
<td>3</td>
<td>Yes</td>
<td>Obligate anaerobes</td>
<td>Bov. &amp; Ov. rumen</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Asterolesma</em></td>
<td>1</td>
<td>No</td>
<td>Obligate anaerobes</td>
<td>Bov. &amp; Ov. rumen</td>
</tr>
</tbody>
</table>


[^b]: Phytoplasmas have an uncertain taxonomic affiliation because they cannot be cultivated.

[^c]: Except some species isolated from cold-blooded vertebrates.
differences can be studied because the molecule contains regions of different evolutionary variability. The size of about 1500 nucleotides is convenient for rapid sequence analysis and the presence of universal regions enable its amplification by PCR for subsequent analysis (68). PCR and sequencing primers can easily be designed to target universal regions of the 16S rRNA genes also from organisms which have not earlier been described or for organisms which cannot be cultivated (64). This possibility has proved very useful for classification of phytoplasmas (59) which are uncultivable. A particularly fascinating recent development has been the reassignment of several uncultivable rickettsias from the genera *Haemobartonella* and *Eperythrozoon* into the genus *Mycoplasma* on the basis of their 16S rRNA similarity (142). Another important advantage of the 16S rRNA gene is that it is not prone to horizontal gene transfer, and that polymorphism between copies of the gene in the chromosome are not common; due to recombination by gene conversion.

The accumulated 16S rRNA sequence data led to the construction of a hypothetical scheme for mollicute phylogeny. Accordingly, the ancestral mycoplasma arose from the *Streptococcus* phylogenetic branch about 605 million years ago. Mollicutes evolved as a branch of gram-positive bacteria by the process of reductive or degenerative evolution. During this process, the mollicutes lost considerable portions of their ancestors’ chromosomes but retained the genes essential for life (124). Approximately 500 million years ago the mycoplasma branch is thought to have evolved and the requirement for sterol originated (Tables 2.1 & 2.2). Interestingly, the UGA codon, which in other prokaryotes (including the earlier-evolved mollicutes) is a stop codon, became reassigned in this group of organisms as a tryptophan codon (Table 2.1) (194). The whole genome sequencing of *Mycoplasma genitalium* and *Mycoplasma pneumoniae* allow an insight to the nature of the reduced genetic information of mollicutes.
During their evolution the two mycoplasmas have apparently lost all the genes involved in amino acid biosynthesis and most of the genes involved in cofactors biosynthesis, and thus require the full spectrum of amino acids and vitamins from the host or the medium. The two organisms also have a reduced set of genes for other metabolic pathways (e.g., lipids, nucleotides), and cellular mechanisms (e.g., energy metabolism, regulatory functions, transport, replication). The considerable economization in genes depends primarily on the obligate parasitic mode of life that was adapted by mollicutes (162).

The Genus *Mycoplasma*

The members of the genus *Mycoplasma* are found in multiple hosts, including humans and many cold and warm blooded animals, and tend to be host specific. Only a relatively small number of the mycoplasma species are known as pathogens, and most species exist as commensals. They are primarily found as surface parasites on mucous membrane surfaces of the respiratory tract and urogenital tracts, as well as joints, eyes and mammary glands (162). Pathogenic mycoplasmas have pronounced affinity to mucous tissues and usually cause a chronic disease with high morbidity and relatively low mortality. Most species reside extracellularly, but some species like *M. pneumoniae*, *M. genitalium*, *M. penetrans*, and *M. gallisepticum* may localize and survive within non-phagocytic cells in-vitro (32, 189). While the wall-less state of mycoplasmas probably contributes to a marked pleomorphy, most species of the genus *Mycoplasma* approach the coccoid state. *Mycoplasma pneumoniae* and its closest phylogenetic relatives (*M. genitalium*, *M. gallisepticum*, *M. imitans*) depart from this norm by having the attachment organelle structure protrusion. The attachment organelle is a large structure with a central rod, into which more than eight proteins are integrated. The organelle best characterized
function is mediation of attachment to host cells (cytadherence), but it is also involved in other essential functions (13, 94).

As mycoplasmas lack a cell wall and have limited metabolic options for replication and survival, these minimal bacteria have developed mechanisms for efficient colonization of the host and for escape from the host immune response. Adhesion of mycoplasmas to epithelial cells is considered to be a prerequisite for colonization and infection. Loss of adhesion results in loss of infectivity, and reversion to a cytadherence phenotype is accompanied by regaining infectivity and virulence (102, 163). Some pathogenic mycoplasmas have a specialized attachment organelle, as mentioned above, which mediate adherence. Some of the attachment organelle proteins appear to be associated with a novel cytoskeleton that is unknown in other prokaryotes (175). The cytoskeleton structure is thought to have an important function in colonizing adhesion, in adjusting the mycoplasma cell shape and also in the gliding motility that occur in some mycoplasmas (134). It may therefore help the mycoplasma to reach and adhere to its target cells, and hence have a fundamental role in pathogenicity.

The main host immune response evasion mechanisms are characterized by the presence of highly mutable modules and an ability to expand the antigenic repertoire by generating structural alternatives within very limited genomic space. The generation of a versatile surface coat allows mycoplasmas to escape the humoral arm of the immune response. Observations over the past 15 years have revealed the extreme variability of mycoplasma cell surface composition among clonal populations (34, 166). Molecular mechanisms involved in this phenomenon are subject to two types of variation, both of which occur spontaneously at a high frequency. The first of these is phase-variation, or ON and OFF switching, where a particular component undergoes variation in expression. The second, commonly referred to as size variation, affects
the structure of these components, in most cases by altering the length of their carboxyl-terminal regions. All the variable cell surface components that have been described to date in mycoplasmas are proteins (mainly lipoproteins) and can be divided into two categories: those encoded by multigene families and those encoded by single genes. Interestingly, the presence of systems generating high frequency phase and/or size variation seems to be restricted to mollicutes that colonize immunocompetent hosts (Mycoplasma and Ureaplasma species) and, so far has not been reported in those infecting plants or insects (Spiroplasma and Phytoplasma species) (30). Other host immune system evasion mechanisms are the reduced ability of macrophages to kill mycoplasmas, and basically the inability of neutrophils to harm them. Most mycoplasmas can be killed by macrophages only with the assistance of opsonins, and in some cases specifically by antibodies. This allows the mycoplasma time to colonize until opsonins will become available. Neutrophils appear not to be effective at all against mycoplasma and their recruitment to the site of infection can only cause more tissue damage, and might help in the dissemination of the infection; some mycoplasmas can be phagocytized but not killed by the neutrophil and travel to other sites (169).

The hallmark of many mycoplasma diseases is the persistence of the organism, and the provocation of frustrated and ineffective immune responses against the infection result in the development of chronic inflammation. Although the complete mechanisms through which mycoplasma infection is able to persist are unknown, it is clear that development of ineffective immune response leads to immunopathology. Immune response can be elicited to protect from infection, and therefore, vaccines can be effective. However, once established, a mycoplasma infection can often persist in the face of an onslaught of an array of immune effector mechanisms (169).
The molecular basis of mycoplasma pathogenicity remains largely elusive. It appears that most of the damage to the host is caused by its own immune response (immunopathology), but additional pathogenesis mechanisms were described. *In-vitro* and *in-vivo* loss of ciliary activity in the respiratory tract were reported (2, 35), and could assist in the mycoplasma colonization. Reactive oxygen species by-products of mycoplasma metabolism, such as superoxide and hydrogen peroxide, may be involved in oxidative damage to host cells membranes and intracellular components during mycoplasma infection (161, 162).

Mycoplasmas are also frequent cell culture contaminants, and the most common cell culture contaminating species are: *M. arginini* *M. fermentans*, *M. orale*, *M. hyorhinis*, *M. hominis*, *M. salivarium*, *M. pirum* (178).

**Avian Mycoplasmas**

Currently there are over 23 avian mycoplasma species, one acholeplasma species (*A. laidlawii*), and one ureaplasma species (*U. gallorale*) described in birds. The species that were recognized as pathogens of domestic poultry include *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in chickens and turkeys, and *Mycoplasma meleagridis* (MM) and *Mycoplasma iowae* (MI) in turkeys. The most updated listing of avian mollicutes could be found on the web site of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/htbin-post/Taxonomy/wgetorg). Avian mycoplasmas have complex nutritional requirements, tend to grow relatively slowly, and are rather resistant to thallium acetate and penicillin, which are frequently employed in media to retard growth of contaminant bacteria and fungi. Nonpathogenic avian mycoplasmas such as *M. gallinarum* and *M. gallinaceum* may grow faster and in many field situations interfere with the isolation of the
slower growing pathogenic species. Within the avian mycoplasmas, the majority of species either ferment glucose or hydrolyze arginine, while a small number have both activities and some have neither (81). Determination of the species of avian mycoplasma isolates could be aided by their biochemical properties (e.g., glucose fermentation, arginine hydrolysis), but direct staining of mycoplasma colonies on agar surfaces with specific fluorescent antibody (171) is most commonly used.

Mycoplasma gallisepticum

MG is the most economically significant avian mycoplasma, and causes respiratory disease in chickens, turkeys and other avian species. The disease, in chickens known as chronic respiratory disease (CRD) and turkeys as infectious sinusitis, can result in rales, coughing and nasal discharge, as well as sinusitis in turkeys. Airsacculitis may cause significant economic losses at processing, and there may also be egg production losses, reduced feed efficiency, medication and surveillance costs (105). Mild or subclinical cases of MG, termed ‘atypical’ infections, have been observed in chickens and turkeys (33, 121, 195). These atypical infections are often difficult to diagnose (10, 72). Turkeys are more susceptible to MG and often more severely affected by MG infections than chickens; turkeys may develop severe sinusitis, respiratory distress, depression, decreased feed intake, and weight loss (105). The clinical manifestation of severe MG infection in chickens and turkeys is generally due to a complicated etiology involving concurrent infections and environmental factors. Colibacillosis, live viral vaccines, and immunosuppression may all affect the severity of the disease (58, 85, 138, 141).

Although MG infection occurs naturally in chickens and turkeys, the organism has also been isolated from naturally occurring infections in other avian species (105). The significance
of these species in the epizootiology of MG has not been established although wild passerine species may act as biological carriers (91, 148). In early 1994, an epidemic of MG began in wild house finches (*Carpodacus mexicanus*) in the mid-Atlantic United States (109). MG had not been previously associated with clinical disease in wild passerine birds. The disease has become widespread and has been reported throughout the United States and Canada (104). Molecular characterization of isolates generally suggested that the house finch epidemic arose from a single source and that the MG infection had not been shared between songbirds and commercial poultry, although one 2001 isolate from New York was clearly different from the other songbird samples and clustered together with the vaccine and reference MG strains, indicating that substantial molecular evolution or a separate introduction has occurred (27).

MG can be transmitted horizontally by direct or indirect contact through the respiratory tract. In general MG does not survive outside of the host for extended periods. It has been shown to survive on straw, cotton, and rubber for up to 2 days and 3-4 days on human hair or feathers (29). Carrier birds, including backyard flocks, are thought to be the main source of MG outbreaks. MG can also be transmitted vertically *in ovo*. The highest frequency of vertical transmission occurs during the acute phase of the disease, but transmission may also occur at a lower rate during chronic infection (54, 55).

Gross lesions consist primarily of catarrhal exudates in nasal and paranasal passages, trachea, bronchi and air sacs. In the acute and subacute phases congested to hemorrhagic tracheitis is usually observed. Sinusitis is most prominent in turkeys but is also observed in chickens and other affected avian hosts. Air sacs frequently contain caseous exudate, and some degree of pneumonias may be observed. In sever cases airsacculitis, fibrinous or fibrinopurulent perihepatitis, and adhesive pericarditis can occur and result in high mortality and extensive
condemnation at processing (105). Conjunctivitis is characteristic of MG infection of house finches and other song birds (109, 133). Microscopic lesions in sinuses consist of degenerating and sloughing of the epithelium, and infiltration of heterophils in the lamina propria. Later, epithelial hyperplasia, mucus gland hyperplasia, and macrophage, lymphocyte and plasma cell infiltration in the lamina propria occur. Lymphoid follicle formation occurs in the lamina propria and submucosa. Similar changes are seen in the trachea and the primary bronchi. Tracheal mucosal thickness, have been used as measures of MG infection and disease (183). Changes in the air sacs are characterized by an increase in air-sac thickness, fibrin exudate, heterophil, lymphocyte and macrophage infiltration, and epithelial cell degeneration and hyperplasia (41).

**Mycoplasma gallisepticum surface molecules.** Epitope switching has been observed for several MG surface molecules (15, 45, 99). Some of these surface molecules are putative cytadhesins. MG cytadhesins-like that have been identified include LP64 (42), VlhA (pMGA) (128, 154), PvpA (18, 199), MGC1 (57, 70), MGC2 (62), and MGC3 (156, 201). Antigenic variations of cytadhesins allow MG to escape the host immune system (8).

The surface variable lipoprotein and haemagglutinin VlhA (previously termed pMGA) is expressed in abundance by MG (129), and encoded by the *vlhA* multi-gene family. It has been well established that by an unknown mechanism MG generally expresses a single member of the family at any one time (53) and that the specific gene expressed can be influenced by growth in the presence of cognate antibody (127). The probable role of this family in generating antigenic variation has been demonstrated in infected chickens, with both phase variation demonstrated during the acute stages of disease, and antigenic switching during the chronic stages (51). These findings have led to the suggestion that the principal function of this family is to generate antigenic diversity and hence facilitate immune evasion during chronic infections. The number
of \textit{vlhA} gene copies present in the genome varies from 32 to 70 in different MG strains (14). In the MG R\textsubscript{low} strain this family contains 43 genes, constituting a total of 103 kb or 10.4\% of the genome. The 43 \textit{vlhA} genes are distributed among five genomic loci containing 8, 2, 9, 12 and 12 genes, respectively (154). The control of transcription of each member of this gene family has been shown to reside in a short GAA trinucleotide repeat motif that lies 18 bases 5’ to the -35 box of the promoter of each gene (52). Slipped strand mispairing resulting from loss or gain of trinucleotide units generates variability within the repeat region. The number of repeats varies between 2 and 27, but only when there are 12 repeats is the gene transcribed and the VlhA protein expressed. The mechanism by which this repeat motif influences the promoter of the gene is yet to be elucidated.

PvpA is a variable surface cytadhesin protein of MG. The \textit{pvpA} gene, present as a single chromosomal copy, encodes a putative cytadhesin related molecule of MG exhibiting high homology to the P30 and the P32 attachment organelle proteins of \textit{M. pneumoniae} and \textit{M. genitalium}, respectively, and high homology to the MGC2 cytadhesin of MG as well (18). The PvpA protein of strain R, shown to undergo variation in its expression, possesses a proline-rich carboxy terminal region containing two identical directly-repeated sequences of 52 amino acids. The molecular basis of PvpA phase variation was revealed in a short tract of repeated GAA codons, encoding five successive glutamate residues, located at the N-terminal region and subject to frequent mutation generating an in-frame UAA stop codon (18). Although this point mutation occurs within an error prone region composed of direct repeated GAA motif, the genetic mechanism underling this event cannot be explained by slipped strand mispairing, which would have resulted in addition or deletion of one or several GAA repeats, as seen in \textit{vlhA} phase variation. In several generations tested, colony immunoblot of the PvpA-positive phenotype
allowed the identification of variants displaying the PvpA-negative phenotype, at a frequency of about $10^{-3}$ to $10^{-4}$ per cell per generation. However, plating of the negative phenotype did not allow the identification of progeny exhibiting the positive phenotype, suggesting that the occurrence of the nonsense mutation is either irreversible or occurs at a low frequency (198). Another type of variation shown to occur with PvpA results from deletions within the 3’ end of the \textit{pvpA} gene and causes size variation of PvpA protein. The size variation of the PvpA protein was shown to range from 48 to 55kDa. The deletions were localized at the proline-rich carboxy-terminal region (18), suggesting that this domain may be under selective pressure in the natural host. Several MG strains differing in their adherence and pathogenicity have varying deletions and sizes of PvpA. Analysis of \textit{pvpA} has been used to differentiate between MG strains (117).

The \textit{gapA} gene (57), also referred to as \textit{mgc1} (70), is one of three clustered genes with adhesin-related functions. It encodes a protein with homology to the P1 attachment organelle protein of \textit{M. pnemoniae}. Immunoblot analysis of various strains has demonstrated intraspecific variation in the size of GapA (98, 105 and 110 kDa) (57). The \textit{gapA} gene product also has been shown to undergoes spontaneous phase variation in expression (188). As for \textit{pvpA}, the basis of this variation is a base substitution that occurs in the amino-terminal coding region of the gene and generates a nonsense mutation leading to premature termination of translation. Reversible switching in expression of GapA was always found to be associated with the same base substitution (AGTTCTTAAT ↔ AGTTCTTTAAAT). The mechanism underlying this event is uncertain, as the sequence surrounding the hot spot for mutation has no particular feature that might suggest the slipped strand mispairing or recombination (188).

The other two genes in the cluster are \textit{mgc2} (62) and \textit{crmA} (156) also referred to as \textit{mgc3} (201). MGC2 is a 32 kDa protein with homology to the P30 and the P32 attachment organelle
proteins of *M. pneumoniae* and *M. genitalium*, respectively. The *mgc2* gene is located next and upstream to the *gapA* gene. CrmA (or MGC3) is a 120kDa cytadherence associated membrane protein sharing significant sequence homology with the ORF6 gene of *M. pneumoniae*, which has been shown to play an accessory role in the cytadherence process. CrmA is cotranscribed with GapA since encoded by the second gene in the *gapA* operon (201). GapA and CrmA interact and are essential for cytadherence (155), and have been shown to undergo concomitant phase variation governed by the *gapA* phase variation mechanism as described above (188).

*Mycoplasma gallisepticum* immunity. Chickens or turkeys that have recovered from clinical signs of MG diseases are known to have some degree of immunity. However, recovered birds may still carry the organism (16) and can transmit infection to susceptible birds by contact or egg transmission. The importance of antibodies and the bursa of Fabricius to the development of resistance and serologic response to the organism has been demonstrated (4, 95), but it has also been shown that there is a poor correlation between systemic antibody levels and protection from respiratory challenge (112, 144). Increasing antibody titers to MG were found in tracheal washing of infected chickens with concomitant decrease in organisms and tracheal lesion scores (28, 192). Recent comparisons between challenged sham-vaccinated and live vaccine vaccinated chickens demonstrated the higher level of MG specific IgA and IgG secreting plasma cells in the tracheal mucosa of protected birds. These studies also demonstrated the massive infiltration of inflammatory cells (CD4+, CD8+, NK cells) in naïve chicken tracheas, versus minimal infiltration in vaccinated bird tracheas, and suggested the role of these cells in the disease pathogenesis (66, 47). The cumulative data indicates that antibodies in respiratory secretions play a major role in resistance to MG. MG antibodies in upper and lower respiratory tract washes have been shown to prevent attachment and establishment of MG in tracheal organ cultures (9)
and in vivo (173, 192), and may be one important mechanism of local antibodies immune-mediated protection. The upper respiratory tract lesions usually resolve in 3 to 7 weeks with a concomitant decrease in MG in the trachea following control of the infection. The resolution of lesions is correlated with increasing antibodies in tracheal washes (192) and serum, and leukocyte migration into the mucosa (28). It has been theorized that local immunity mediated by secretory IgA may have a role in preventing the establishment of infection while CMI may be involved in recovery (173). The presence of maternal antibodies to MG in embryonated eggs reduced the in ovo pathogenicity of infection and increase the probability of survival of the infected embryo (101). Birds lacking a fully functional immune system (neonatal, thymectomy or bursectomy) have significantly higher tracheal lesion scores and organism counts than normal birds following MG infection (48, 173).

**Mycoplasma gallisepticum control.** Control of MG has generally been based on the eradication of the organism from breeder flocks and the maintenance of mycoplasma-free status in the breeders and their progeny by biosecurity. Single-age and all-in all-out production methods allow the control of MG in this way. Serology is the primary method for flock screening. Serological monitoring performed periodically is the basis of voluntary control programs such as the US National Poultry Improvement Plan (NPIP). Large populations of poultry in small geographic areas can make control by biosecurity alone very difficult. MG vaccines have been used in the control of MG in areas where eradication is not feasible. MG vaccines are used to prevent or reduce disease and clinical signs in the vaccinated birds as well as to prevent egg production losses and egg transmission of MG.

Inactivated MG vaccines have been widely used in several countries. The results with MG bacterins have been variable; some investigators found that bacterins could protect broilers
from airsacculitis (69, 197) or layers from reduction in egg production (61, 196), and others did not detect much efficiency in commercial egg layers with endemic MG infection (79). Vaccination with bacterins has been shown to reduce, but usually not eliminate, colonization by MG following challenge (90, 170) and generally are felt to be of minimal value in long-term control of infection on multiple-age production sites (97).

Recently, Biomune (Lenexa, KS) developed a recombinant Fowlpox virus vaccine that was cloned with 2 MG surface proteins: the cytadherence associated membrane protein MGC3 (also referred as CrmA) and the VlhA-like lipoprotein MGR 365 (201, 168). This vaccine trade name is VECTORMUNE® FP-MG and is licensed in the US for MG + Fowlpox vaccination.

One of the options for control is live MG vaccines (86, 97, 184). Eradication of MG is preferable to vaccination wherever possible; however, the use of live vaccines to displace virulent wild-type MG strains from commercial poultry flocks may be a useful part of an eradication program (84, 103). Live vaccines that are currently used worldwide to control MG include F strain (Schering Plough, Kenilworth, N.J.) (5, 120), 6/85 (Intervet America, Millsboro, Del.) (36) and ts-11 (Bioproperties, Inc., Australia, marketed in the US by Merial Select Laboratories, Gainsville, GA.) (185, 186). The important characteristics of an ideal live MG vaccine include safety in the target species, efficacy (immunogenicity), the ability to stimulate solid lifelong protection (preferably from a single dose), and stability following in vivo passages (lack of reversion of attenuated strains to a virulent form). Vaccines should also be easy and inexpensive to manufacture. The vaccine should not spread to neighboring flocks (184), and needs to be differentiable from field strains. It has been established that there is a complex relationship between infectivity, pathogenicity and immunogenicity of MG strains (102). It has been established that virulence, invasiveness and immunogenicity of MG strains are directly
correlated (114). Some live vaccines may be so attenuated as to be incapable of eliciting long lasting protective immunity. The colonization and persistence of MG in the upper respiratory tract may be essential to duration of immunity elicited by the vaccine. Studies have indicated that the level of protection elicited by live vaccines is directly correlated with the virulence of the vaccine strain (1, 113).

F strain is a relatively mild strain that originated from a pathogenic field isolate (193) that was further studied by van der Heide (179). F strain vaccine has been used extensively in multiple-age laying complexes to reduce MG-caused egg production losses (1, 20, 21, 31, 54, 55). F strain vaccinated laying hens produced more eggs than unvaccinated hens in flocks with endemic MG but not as many as MG-clean flocks (25, 135). The mechanism underlying protection by F strain was found not to involve competition with the challenge strain for adherence sites or blockage by prior colonization (100). F strain vaccinated flocks maintain the organism for life and can transmit it through the egg (115) and among penmates (92, 36). F strain is very immunogenic and mildly virulent in chickens (1, 20, 165), but too virulent for use in turkeys (116). F strain was found to be pathogenic in turkeys following experimental infection (116) and it has been associated with MG outbreaks in commercial turkeys (110). F strain is effective in displacing virulent (field) strains from poultry operations (87, 84, 103). The vaccine is safe for young chicks and can be administered as early as 2 weeks or less (97).

The 6/85 strain of MG oriented in the US, and its development and vaccine characteristics were described by Evans et al. (36, 37). Studies using MG 6/85 vaccine found minimal virulence in chickens and turkeys, little or no transmissibility from bird to bird, and resistance against challenge with virulent MG (1, 36, 107).
Development and characterization of the ts-11 MG vaccine have been described by Whithear et al. (185, 186). The ts-11 vaccine strain originated from an Australian MG field isolate that was subjected to chemical mutagenesis and selected for temperature-sensitivity (growth at 33°C) (186). The ts-11 MG vaccine has minimal or no virulence for chickens and turkeys, is weakly transmissible from bird to bird, persist in vaccinated flocks for life, and induces protection to MG experimental and field challenges (1, 107, 184, 185, 186).

The ts-11 and 6/85 vaccines have both been shown to be poorly transmissible to in contact poultry (107, 186). The distinct advantage of the milder vaccine strains over F strain is their lack of virulence in turkeys and their low transmissibility (36, 107, 116, 186). F strain persists at higher levels in the upper respiratory tract than either ts-11 or 6/85, and ts-11 appears to colonize more effectively than 6/85 (1, 107). F strain may be more virulent than 6/85 or ts-11, but it provides better protection against airsacculitis (1), and protects against colonization by more virulent challenge strains (31). F strain vaccination was shown to displace virulent MG strains, whereas 6/85 or ts-11 vaccine strains did not (84, 87). However, the persistence and transmissibility of F strain allows its continued cycling in vaccinated farms long after vaccination has ceased. The ts-11 vaccine may be useful in displacing endemic F strain in poultry complexes as part of an eradication program (177). Although, in experimental situations, F strain has been shown to transmit between birds, widespread use of the vaccine has not resulted in widespread isolations of F strain from field cases in chickens (50). In the event that a live vaccine cycles through a flock of poultry it should not increase in virulence. After years of use there is no evidence that F strain has become more virulent (184). Experimental passage of 6/85 through chickens and turkeys did not result in a substantial increase in virulence (36, 37).
Attempts to serially passage ts-11 in birds were unsuccessful but the vaccine appeared to retain its characteristics after three passages (186).

The vaccination of turkeys against MG has not been shown to be feasible although there has been limited use of 6/85 (97). A high passage (164 times) MG R strain mutant (GapA-, CrmA- and HatA-) with the gapA gene complementation, designated GT5, has recently been described as a potential modified live vaccine (66, 155, 156). The characteristics of different live MG vaccines have been described and compared extensively (1, 107, 184). The choice of vaccine should be carefully evaluated in each situation.

*Mycoplasma synoviae*

*Mycoplasma synoviae* (MS) is an important pathogen of domestic poultry, causing economic losses to the poultry industry (82). Infection most frequently occurs as a subclinical upper respiratory infection, which can progress to respiratory disease with air sac lesions when exacerbated by other respiratory pathogens (e.g., Newcastle disease virus, infectious bronchitis virus), or when more virulent MS strains are involved (93, 118). The infection can also become systemic and result in infectious synovitis, an acute to chronic infectious disease of chickens and turkeys, involving the primarily the synovial membranes of joints and tendon sheaths producing an exudative synovitis, tenovaginitis, or bursitis (82). Infectious synovitis was first described and associated with mycoplasma by Olson et al. (151, 152).

MS requires nicotinamide adenine dinucleotide (NAD), and cysteine hydrochloride as the NAD reducing agent, in addition to the complex nutritional requirements of avian mycoplasmas. Chickens, turkeys, and guinea fowl are the natural hosts of MS, but ducks, geese, pigeons, house sparrows, pheasants, and Japanese quail may also be susceptible. MS is present in most poultry-
producing countries, and infection occurs frequently in multi-age commercial layer complexes (136, 153). Transmission may be transovarian, or lateral via respiratory aerosols and direct contact. Lateral transmission occurs via the conjunctiva or the upper respiratory tract, and usually 100% of the birds become infected. Vertical transmission plays a major role in the spread of MS in poultry. Following infection birds become persistently infected with MS, and remain carriers for life.

There is considerable variation among isolates in their ability to cause disease, and many isolates cause little or no clinical disease (118, 119). The pathogenicity mechanisms of MS involve attachment and colonization of the upper respiratory tract plus additional unidentified factors associated with systemic invasion and lesion production. MS isolates from air sac lesions are more apt to cause airsacculitis, and those isolated from synovia are more apt to produce synovitis (93).

*Mycoplasma synoviae surface molecules.* MS has two major surface antigens, MSPA and MSPB, and it has shown that MSPA is a haemagglutinin and MSPB is a lipoprotein (147). These antigens are both encoded by a single gene, *vlhA* (variable lipoprotein and haemagglutinin), possibly with post-translational cleavage generating the two proteins. The coding sequence of the *vlhA* gene is homologous to those for the *vlhA* genes of MG, even though MS is phylogenically distant from MG (146). Like VlhA (previously termed pMGA) in MG the haemagglutinin MSPA is subject to high frequency phase and antigenic variation (147). In spite of the coding similarities, the mechanism used to generate variation in the expressed MS *vlhA* gene is quite distinct from that controlling the MG *vlhA* gene repertoire (145). There is only one transcriptionally and translationally competent *vlhA* gene in the MS genome. However, while there is a single copy of the promoter and the first 408 bases of the coding sequence, there are
multiple, variant copies of the region encoding the 3’ end of the gene. These pseudogenes appear to be clustered within a restricted region of the genome as tandem repeats (7), and seem to control the antigenic variation in MS by multiple gene conversion events.

*Mycoplasma synoviae control.* Following the successful use of chemical mutagenesis to create the ts-11 strain of MG, Morrow et al. (137) used the same procedure to produce temperature sensitive clones of an Australian field isolate of MS for assessment as potential vaccine. A strain designated MS-H expressed the $ts^+$ phenotype, colonized the trachea of chickens and elicited serum antibody response 3 weeks after eye-drop inoculation. Subsequent evaluation of the efficacy (125) and safety (126) of both laboratory produced and commercially manufactured MS-H vaccine confirmed that it was efficacious and safe. Following eye-drop administration, the MS-H vaccine colonized the upper trachea of turkeys, elicited a detectable serum antibody response, but caused no lesion in the respiratory tract (143).

**Diagnosis of Mycoplasma gallisepticum and Mycoplasma synoviae**

Serological screening is routinely used as an indicator of MG and MS infection. Sera commonly are analyzed for antibodies using the serum plate agglutination (SPA) test, a hemagglutination-inhibition (HI) test and an enzyme-linked immunosorbent assay (ELISA) test (83). The SPA test is rapid, sensitive and inexpensive but may result in nonspecific reactions (3, 19, 56). These have been related to medium components, primarily serum, adhering to surface of the mycoplasma organism used to prepare the antigen, although false positive reaction may often be unexplained (12). False positive reactions are commonly seen after chickens or turkeys have been vaccinated with inactivated oil emulsion vaccines against other infectious agents, especially if there are remnants of serum in the vaccine (56). Such false positive reactions may persist up to
4-8 weeks or longer after vaccination. Production of agglutination antigens in medium substituting artificial liposomes for serum may result in agglutination antigens of improved specificity (6). In addition several cross reacting antigens between MG and MS are known to occur (11). The SPA test may also yield false negative results in some instances; Ewing et al. (38) reported that the MS SPA test missed infected commercial layer and breeder flocks that were detected by MS ELISA.

SPA reactors must generally be confirmed by the HI or ELISA tests. The HI test is less sensitive but more specific than the SPA test. It is however, a longer procedure and the reagents are not commercially available. In general the ELISA test is more sensitive than the HI test and more specific than the SPA test (71, 74). Infection is generally confirmed by the isolation and identification of the organism or by DNA based detection methods (97). Isolation and identification of the organism is generally considered the gold standard for diagnosis. For culture swabs from trachea, choanal cleft or air sacs are often used. Sinus exudates, as well as swabs of the turbinates, and lungs and other tissues may also be used (83). Mycoplasma isolates are commonly identified using direct and indirect immunofluorescence (83, 171). Mycoplasma species-specific hyperimmune sera is an essential reagent for these tests and may limit the ability of some laboratories to perform the test (83). MG and MS species-specific PCR (96, 140), MG real-time PCR (24, 132), PCR-RFLP (44, 80) and oligonucleotide probe (40, 43) techniques have been developed. During the acute stage of the infection the number of organisms in the upper respiratory tract is high (100, 192); however in chronic infection the number of organisms is much lower and routine methods may not detect it (97). In some situations it may be very difficult to isolate pathogenic mycoplasma consistently from infected flocks. These instances include chronic MG and MS cases and infections with strains of low pathogenicity (83, 195).
The overgrowth of non-pathogenic mycoplasmas may also interfere with cultivation of pathogenic mycoplasmas from clinical samples in the laboratory (123). Recently a method to separate rapidly growing nonpathogenic avian mycoplasma species from slower-growing MG field strains was reported (17). Mixtures of MG and nonpathogenic avian mycoplasmas were inoculated onto chick embryo fibroblasts cells (CEF) allowing MG to penetrate the CEF cells. Later, gentamicin sulphate was added to the culture, eliminating the nonpathogenic mycoplasmas and allowing MG to be isolated in pure culture. The isolation rates of fastidious MG strains may be enhanced \textit{in vivo} by bioassays (122). Susceptible poultry are inoculated with potentially infectious material from suspect flocks. The organism may have the opportunity to multiply in these birds to levels detectable by PCR and/or culture. The birds are routinely sampled enhancing MG detection.

\textbf{Epizootiology and Strain Differentiation in Avian Mycoplasmolgy}

In general the process of subtyping microbial isolates into strains is important epidemiologically for recognizing outbreaks of infection, determining the source of the infection, recognizing particularly virulent strains of organisms, and monitoring vaccination programs (149). Methods of strain differentiation must have high discriminatory power so that it can clearly differentiate unrelated strains, as well as demonstrate the relationship of isolates from individuals infected through the same source. The techniques should also have a high degree of reproducibility. Reproducibility refers to the ability of a technique to yield the same result when a particular strain is repeatedly tested. It is especially important for the construction of reliable databases containing known strains within a species to which unknown organisms can be compared. Mycoplasma colonies can vary in their surface antigenic phenotype, therefore
mycoplasma strains can differ markedly in their antigen profiles and their potentially virulence-related surface properties (167). Intraspecies heterogeneity and antigenic variability can be observed in mycoplasmas through serological testing (88, 172) and electrophoresis of cell proteins (78). The shortcomings of phenotypically based typing methods, such as those based on a reaction with an antibody (167), have led to the development of typing methods based on the microbial genotype or DNA sequence, which minimize problems with typeability and reproducibility and, in some cases, enable the establishment of large databases of characterized organisms. Molecular techniques that have been used to identify avian mycoplasma strains were restriction fragment length polymorphisms (RFLP) of DNA (76, 89), amplified fragment length polymorphism (AFLP) (63), pulsed-field gel electrophoresis (PFGE) (130, 131), DNA and ribosomal RNA gene probes (77, 200), and PCR with strain-specific primers (139). The most widely used method for strain differentiating is random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR, analysis (26, 39, 49). The RAPD assay was first described by Williams et al. (187) and Welsh and McClelland (182). RAPD assays are based on the use of short random sequence primers, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures so that they initiate amplification of regions of the bacterial genome. The number and location of these random primer sites vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis, a pattern of bands results. In theory, the patterns of bands are characteristic of the particular bacterial strain. RAPD analysis is rapid and sensitive and this method has been used to identify vaccine strains in MG-vaccinated flocks and for epizootiological studies (73, 98, 106, 108). Due to the random nature of the primers and the low-stringency conditions of the RAPD reaction, this assay requires the use of pure cultures of the target mycoplasma. Isolation of mycoplasma is
expensive, time-consuming, and technically complicated in cases where nonpathogenic mycoplasma species may overgrow the virulent mycoplasma. The isolation process itself may favor the growth of one strain where more than one subtype may be present. Furthermore, technical factors such as template DNA to primer ratio may significantly impact the reproducibility of RAPD patterns.

Ultimately, all molecular genetic methods for distinguishing organism subtypes are based on differences in the DNA sequence. Logically, then, DNA sequencing would appear to be the best approach to differentiating subtypes. DNA sequencing generally begins with PCR amplification of a sample DNA directed at genetic regions of interest, followed by sequencing reactions of the PCR products. DNA sequencing must be directed at a small region of the bacterial genome. It is impractical to sequence multiple or large regions of the chromosome. Thus, in contrast to RAPD analysis, which examines the entire chromosome, DNA sequencing examines a very small portion of the sites that can potentially vary between strains. The variability within the selected sequence must be sufficient to differentiate different strains of a particular species. Progress in the molecular biology of mycoplasmas has been achieved in the last decade, and several surface proteins in virulent mycoplasmas, such as PvpA (18, 199), MGC1 (57, 70), MGC2 (62), MGC3 (156, 201), and MS VlhA (145) have been described. The DNA sequences of these genes are under great selective pressure and may be useful in the molecular epidemiology of MG and MS.
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CHAPTER 3

ROLE OF *MYCOPLASMA SYNOVIAE* IN COMMERCIAL LAYER E. COLI PERITONITIS

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Role of *Mycoplasma synoviae* in Commercial Layer *E. coli* Peritonitis Syndrome

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**ABSTRACT.** *Mycoplasma synoviae* (MS) is an important pathogen of domestic poultry, and is prevalent in commercial layers. During the last decade *Escherichia coli* (*E. coli*) peritonitis became a major cause of layer mortality. The possible role of MS in the laying hens *E. coli* peritonitis syndrome was studied. Four groups of 64 mycoplasma free commercial layers at the onset of lay (about 80% daily production) were challenged with a virulent MS strain or a virulent avian *E. coli* strain, or both. The 4 experimental groups were: negative control, *E. coli*, MS, and MS plus *E. coli*. A typical *E. coli* peritonitis mortality was reproduced and included 1, 3, 0, and 5 birds in the negative control, *E. coli*, MS, and MS plus *E. coli* groups, respectively. Only the increased mortality in the MS plus *E. coli* group had statistical significance. Four weeks post challenge 10 clinically normal birds from each of the four experimental groups were necropsied. All the examined birds in the two MS challenged groups demonstrated severe tracheal lesions. Body cavity lesions were detected in 2 and 4 birds in the MS and MS plus *E. coli* groups, respectively. The results demonstrate a possible pathogenesis mechanism of respiratory origin to the layer *E. coli* peritonitis syndrome, show the MS pathological effect in layers, and suggest that a virulent MS strain can act as a complicating factor in the layer *E. coli* peritonitis syndrome.
Key words: *Mycoplasma synoviae*, commercial layers, *Escherichia coli*, peritonitis.

Abbreviations: CCU = color changing units; CFU = colony forming units; *E. coli* = *Escherichia coli*; HI = Hemagglutination inhibition; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; PCR = polymerase chain reaction; PFGE = pulsed-field gel electrophoresis; SPA = Serum plate agglutination.
INTRODUCTION

*Mycoplasma synoviae* (MS) is an important pathogen of domestic poultry, causing economic losses to the poultry industry (10). Infection most frequently occurs as a subclinical upper respiratory infection, which can progress to respiratory disease with air sac lesions when exacerbated by other respiratory pathogens (e.g., Newcastle disease virus, infectious bronchitis virus), or when more virulent MS strains are involved (12, 14). The infection can also become systemic and result in acute to chronic infectious synovitis (10). Transmission may be transovarian, or lateral via respiratory aerosols and direct contact. Infection occurs via the respiratory tract and usually affects 100% of the birds. Following infection birds become persistently infected with MS, and remain carriers for life. Due to the expansion of poultry production and concentration of large, multiage production complexes in restricted geographic area, it is becoming more and more difficult to maintain flocks free of MS.

The role of MS in egg production of commercial layers is questionable. Previous studies have revealed that MS infection is rather common in commercial layers (5, 8, 16). In the US table egg industry, most pullets are MS free in the rearing phase but become infected with MS after their introduction into the multiage laying complexes. The economic impact of MS infection in layers is usually estimated to be low because little or no effect either on egg production (3, 17) or egg quality has been reported (3, 15).

During the last decade *Escherichia coli* (*E. coli*) peritonitis has been considered to be a major cause of layer mortality in the US and other countries (Kenton Kreager, personal communication, 18). Layer *E. coli* peritonitis is characterized by acute mortality without prior morbidity or significant impact on egg production. Peritonitis with yolk material deposited in the body cavity and polyserositis are the two main necropsy observations associated with the
disease. The occurrence of *E. coli* peritonitis in layer flocks appears to have two mortality peaks along the laying cycle. The first increase in mortality occurs around the peaking period in egg production, and suspected to be of respiratory origin. The second increase in mortality is observed during the late lay period at the age of fifty weeks and onward, and suspected to be of vent origin due to vent trauma (Kenton Kreager, personal communication). The increase in mortality due to the *E. coli* peritonitis which occurs during the peaking period varies between few tenths of percent to over one percent per week in severe cases. In one survey, the cumulative mortality in twenty affected layer flocks during this period ranged from 0.26% to 1.71% per week, while mortality in twenty unaffected flocks ranged from 0.07% to 0.30% per week (18). Field impressions suggested that some layer flocks suffer from the peaking period *E. coli* peritonitis mortality simultaneously with the point in time when they turn serologically positive for MS. Scientific reports on layer *E. coli* peritonitis are scarce, but in a flock survey conducted by Vandekerchove *et al.* no association could be found between outbreaks of *E. coli* peritonitis mortality and infection with MG, MS or other avian respiratory pathogens (19).

This study is the first controlled experiment that addresses the query whether MS challenge at the onset of lay could be associated with *E. coli* peritonitis mortality during the peaking period. We also measured the effect of MS challenge during the peaking period with, or without *E. coli* challenge on a variety of egg production parameters.

**MATERIALS AND METHODS**

**Animals, housing and management.** Two hundred and fifty six Hy-line W-36 female pullets were received from a commercial rearing farm at the age of 14 weeks. The chickens were randomly allotted into 4 equal groups of 64 birds and housed in 4 separate identical isolation
rooms. The chickens were placed into commercial layer cages (61cm deep X 61cm width X 35.5cm height) in a stocking density of 8 birds per cage, and each isolation room contained 8 cages. The cages were equipped with nipple drinkers, manual feed trough, and an egg gutter in front of the cage. The pullets were transferred from open houses into light proof isolation rooms, and were kept on the natural day length at that time of the year (14 hours) until 18 weeks of age. From that point ½ hour light increments were given on a weekly basis until reaching a day length of 16 hours. The birds were fed ad libitum with standard layer diet through the experiment. Eggs were collected daily between 8:00 and 9:00 AM.

**Experimental design.** Each of the 4 isolation rooms with 64 birds served as an experimental treatment group. The experimental groups were further allotted into 8 subgroups of 8 birds per cage. Each cage was labeled and monitored separately to receive 8 replicates for each experimental treatment. The four experimental treatments were: negative control, *E. coli* challenged, MS challenged, and MS plus *E. coli* challenged groups. The MS challenge was given when the daily egg production of all groups reached about 80% at the age of 27 weeks. The negative control group received no challenges. The *E. coli* group was challenged with the avian *E. coli* V9 strain (described below) intratrachealy in a dose of 9.5x10^8 colony forming units (CFU) per bird. The MS group was challenged with the MS K3344 strain (described below) by aerosol in a dose of 4x10^8 color changing units (CCU) per bird. The MS plus *E. coli* group was challenged with the MS K3344 strain by aerosol in a dose of 4x10^8 CCU per bird, and three days later was challenged with the avian *E. coli* V9 strain intratracheally in a dose of 9.5x10^8 CFU per bird. The MS challenge was performed on the same date for the MS and the MS plus *E. coli* groups, and the *E. coli* challenge was performed on the same date for the *E. coli* and the MS plus *E. coli* groups.
Two birds from each subgroup (cage) were tested for MG and MS upon arrival at the experimental facility in the University of Georgia, at the point of the MS challenge, and 3 weeks post the MS challenge. At the point of the experiment termination, 4 weeks post MS challenge, 10 birds per group were necropsied, monitored for mycoplasma, and examined for gross and microscopic lesions in the body cavity and trachea.

**Challenge organisms.** The MS K3344 strain from our laboratory archive was isolated from commercial layers with a history of egg production drops, and was shown to be virulent and produced airsacculitis in birds that were experimentally challenged (Fan and Kleven, unpublished, 4). For inoculation, 36 hr broth cultures were prepared in Frey modified medium (11).

The avian *E. coli* V9 strain was received from Dr. R. E. Wooley in the Department of Infectious Diseases, University of Georgia. This strain was isolated from a trachea of a chicken with colibacillosis, and was characterized as virulent by several parameters (7). For inoculation, 18-24 hr broth cultures were prepared in tryptic soy broth medium (13).

**Mycoplasma isolation and identification.** Tracheal swabs from bird samples and from all the mortality were submitted for mycoplasma culture. They were inoculated in Frey modified broth and agar and incubated at 37 C. Mycoplasma isolates were identified by direct immunofluorescence (11).

**General bacteriology.** Bone and visceral organs from all the dead birds through the experiment were submitted for standard bacteriological culture. Specimens were aseptically swabbed and plated on blood and MacConkey’s agars and incubated for 14-18 hr at 37 C under aerobic conditions. Recovered *E. coli* isolates were identified as described (13).
Serum plate agglutination (SPA) test. Serum from bird samples were screened for MS and MG using the Intervet (Millsboro, DE) antigens according to the manufacturer’s instructions. Agglutination was scored on a scale 0 to 4; a score of 1 was considered suspect, and a score of 2 or greater was considered positive.

Hemagglutination inhibition (HI). Serum from bird samples were screened for MS and MG by the HI tests as described (11). Antigens were prepared from MS laboratory strain WVU 1853 and MG strain A5969. Titers of 1:40 were considered suspect, and titers of 1:80 or higher were considered positive.

DNA extraction and polymerase chain reaction (PCR). Choanal cleft swabs from 3 birds were pooled in 1 ml of PBS. Genomic DNA was extracted from 200 μl of the PBS using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) following the manufacturer’s recommendations. PCR amplification of the Mycoplasma gallisepticum (MG) mgc2 gene was performed following the protocol published by Garcia et al. (6). The PCR amplification of the MS vlhA gene (variable lipoprotein and hemagglutinin) was performed following the protocol published by Hong et al. (9). The PCR products were separated on a 2% agarose gel containing 1 mg/ml ethidium bromide and visualized by UV transillumination.

MS vlhA sequence analysis. Prior to sequencing, the MS vlhA PCR products were purified using a QIA PCR purification kit (Qiagen, Inc., Valencia, CA). The nucleotide sequencing was performed at the Integrated Biotechnology Laboratories, located at the University of Georgia, Athens, GA, using automated cycle sequencing. The PCR amplification product was sequenced in both directions with the forward and reverse amplification primers. Raw sequence data was analyzed with the EditSeq program (in Lasergene; DNASTAR, Inc., Madison, WI). The assembly of sequence contigs and multiple sequence alignments were
performed with sequencing project management (SeqMan) and multiple-sequence alignment (MegAlign) programs, respectively (DNASTAR; Lasergene, Inc., Madison, WI).

**E. coli Pulsed-Field Gel Electrophoresis (PFGE).** Agarose-embedded bacterial genomic DNA was digested with 10 U of restriction enzyme *Xba*I overnight at 37°C, and DNA fragments were separated by PFGE (2) in a 1% PFGE agarose gel (BioRad; Hercules, CA) with a CHEF DR-II electrophoretic apparatus (BioRad; Hercules, CA). Electrophoresis was for 25 h with a voltage of 200 V and a linearly ramped pulse time of 2.2 to 54.2 s (2). Lambda DNA Ladder (BioRad; Hercules, CA) served as MW markers.

**Production and mortality monitoring.** All the eggs laid during the experiment were collected. Egg production, egg and shell quality (double yolks, shell less, abnormal shape, thin, cracked), and egg weights were recorded on a weekly basis. All mortality was necropsied, gross pathology was recorded, and samples were taken for general bacteriology, mycoplasma culture and histopathology.

**Tracheal mucosal thickness analysis.** Tracheal lesions were evaluated microscopically by measuring the thickness of the tracheal mucosa. A section of the upper third of the trachea (approximately 2cm distal from the larynx) was fixed in 10% neutral buffered formalin. The tracheal mucosa thickness was measured at four equidistant points on histologic slides of cross sections of tracheas (20).

**Statistical analysis.** A chi-square test for equality of proportions was performed to measure the statistical significance of the mortality and the body cavity lesion results. The analysis was performed with SAS PC (SAS institute Inc. Cary, NC). The mean tracheal mucosa thickness was analyzed by the Tukey–Kramer HSD test (JMP_ Statistics Made Visual; SAS Institute, Inc., Cary, NC). A *P*-value of ≤0.05 was considered significant in all the analyses.
RESULTS

**Mycoplasma monitoring.** The mycoplasma status of the study’s 4 experimental groups was tested by mycoplasma culture and MG and MS: SPA, HI, and PCR; the MS test results are summarized in Table 3.1. All groups were negative to mycoplasma on the initial screening and at the point of the MS challenge. The negative control group remained negative to mycoplasma through the experiment. The MS and the MS plus *E. coli* groups were positive to MS on the 21\(^{st}\) and 28\(^{th}\) days post challenge examinations. All the groups remained MG negative through the experiment.

The *E. coli* group had a low level of MS positive reactors by culture and PCR with negative serology (Table 3.1) on the 21\(^{st}\) day post challenge, suggestive of an early MS infection. On the 28\(^{th}\) day post challenge the number of positive culture and PCR reactors increased, and MS antibodies were detected by SPA but not by the HI test. We assume that this unintentional introduction of MS to the *E. coli* group occurred due to the break in biosecurity during the heat stroke incident (described below).

**Mortality analysis.** During the first 5 weeks of egg production (18 to 23 weeks of age) prior to challenge two spontaneous mortality cases were recorded in the experiment, one in the *E. coli* group and the other in the MS plus *E. coli* group. The cause of death in these two cases was *E. coli* septicemia and peritonitis. An increase in mortality was observed following the avian *E. coli* challenge. The post challenge mortality results are summarized in Tables 3.2 and 3.3. *E. coli* peritonitis was the sole cause of death in all the post challenge mortality. The prominent gross lesions in all the mortality were: dehydration, pericarditis, perihepatitis, thoracic airsacculitis, peritonitis with free yolk and/or caseous material in the body cavity, and oophoritis.
*E. coli* was the only organism isolated from the bone marrow and visceral organs of all the mortality cases. *Mycoplasma synoviae* was isolated from tracheas of 3 out of the 5 mortalities in the MS plus *E. coli* group; the two other cultures from this group were contaminated. All the three mortalities in the *E. coli* group were negative for mycoplasma. Between the evening of the 10th day and the morning of the 11th day post challenge a cooling system failure occurred and led to massive heat stroke mortality in all the experimental groups (Table 3.2). When the problem was detected all the facility doors were opened to allow natural ventilation and technicians came in to fix the cooling system. The surviving birds were not handled and remained in their original cages.

The chi-square test statistical analysis indicated that the MS plus *E. coli* group mortality was significantly higher than the negative control group mortality. The *E. coli* group mortality was not significantly higher than the negative control group mortality. But, the MS plus *E. coli* group mortality was not significantly higher than the *E. coli* group mortality.

**Egg production parameters.** The monitored egg production and egg quality parameters did not show any noticeable differences between the experimental groups.

**Comparison between challenged and isolated organisms.** Sequencing of the MS *vlhA* PCR product were performed on the following samples: the three MS isolates from the MS plus *E. coli* group mortality, two MS isolates from each of the MS positive groups (*E. coli*, MS and MS plus *E. coli*) that were recovered during the final necropsy, and the challenge strain K3344. All the MS *vlhA* sequences were aligned and were found 100% identical to each other and to the challenge strain K3344.

The *E. coli* isolates from the post challenge mortality cases were compared to each other and to the avian *E. coli* V9 challenge strain by PFGE. All the analyzed isolates had identical
patterns and were identical to the challenge strain (Figure 3.1). That includes the *E. coli* isolate from the one mortality in the negative control group.

**Final necropsy and tracheal mucosal thickness analysis.** Four weeks after the MS challenge 10 clinically normal birds per group, labeled randomly from 1 to 10, were necropsied and examined for gross lesions in the body cavity (Table 3.4). Body cavity lesions were detected in 2 and 4 birds in the MS and MS plus *E. coli* groups, respectively, with more prominent lesions in the MS plus *E. coli* group. No bird in the negative control group or the *E. coli* group demonstrated body cavity lesions. Statistical analysis of the results indicated that only the lesions in the MS plus *E. coli* group were statistically significant different from either the negative control or the *E. coli* groups. Microscopically, the major lesions in the affected birds were chronic lymphocytic air-sacculitis and granulomatous serositis with multinucleated giant cells.

During the necropsy tracheas were collected for thickness analysis from each of the necropsied birds (Table 3.4). The two MS challenged groups (MS and MS plus *E. coli*) had similar tracheal thickness results and were statistically significant higher than the tracheal thickness of the negative control group. The differences in tracheal thickness between the two MS challenged groups and the accidentally infected *E. coli* groups were numerically obvious but not statistically significant. The differences in tracheal thickness between the negative control group and the *E. coli* group were not statistical significant. The microscopic nature of the tracheal lesions in all the affected groups was similar and was defined morphologically as lymphocytic tracheitis.
DISCUSSION

*E. coli* peritonitis is a common cause of commercial layer mortality and believed to be the number one cause of layer mortality in the US table egg industry during the last decade (Kenton Kreager, personal communication). Layer *E. coli* peritonitis is considered to be normal when it occurs at levels of about 0.1% per week or less (18). Also in this experiment 2 layers died during the first 5 weeks of production to generate an average mortality of 0.15% per week, which could be interpreted to be within the normal range. It is important to realize that the concern about the syndrome is due to the higher incidence and not the syndrome occurrence by itself. In order to investigate the possible causes for the increased level of layer *E. coli* peritonitis we had to develop a model that would reproduce the syndrome. The successful reproduction of a typical layer *E. coli* peritonitis disease by the experimental model allowed us to investigate the role that MS might have in this syndrome. A beneficial byproduct of the experimental model was the suggestion of a pathogenesis mechanism for the disease. The direct application of the causative *E. coli* to the upper trachea and the reisolation of an identical *E. coli* strain from the viscera and bone marrow of dead birds with peritonitis are highly suggestive of the respiratory origin of the layer peritonitis syndrome. This observation is in agreement with the industry notion of the respiratory origin of layer peaking period *E. coli* peritonitis (Kenton Kreager, personal communication).

The severity of the tracheal lesions that were induced by the K3344 MS strain brings into question the prevailing assumption of MS as a benign infection in layers. Indeed, the affected chickens appeared clinically normal, but it is likely that birds with such altered tracheas will be more susceptible to respiratory disease agents and environmental contaminants. Our hypothesis
is that the injured trachea would allow the avian *E. coli* higher level of colonization and systemic penetration that would result in a higher level of peritonitis.

The experiment demonstrated that the combined challenge of MS plus *E. coli* induced statistically significant mortality relative to the negative controls while the *E. coli* alone did not. That result implies that the intratracheal *E. coli* challenge by itself is not enough to produce significant peritonitis mortality in layers and that the MS challenge was required to induce the peritonitis mortality. This finding is in agreement with the general notion of colibacillosis as a secondary disease, following a primary infection with respiratory pathogens and/or unfavorable environmental conditions (1). But the above interpretation appears less conclusive when considering the only numerical, and not statistically significant, difference between the mortality in the *E. coli* and the *E. coli* plus MS groups. The difficulty to achieve a more definitive conclusion was the consequent of the relatively low *E. coli* peritonitis mortality in our model, previous models (21), and most clinical cases, and it is questionable whether mortality is the statistically appropriate measurement for this syndrome. The necropsy observation of abundant sub-clinical lesions in the body cavity of MS challenged birds versus no lesions in the solely *E. coli* challenged group is supportive of the significant role of the MS challenge in the layer peritonitis syndrome. It also leads us to think that body cavity sampling (lesion scoring, histology, *E. coli* culture), from 7 days post challenge and onward, could be a better tool for measuring the effect of the different challenges utilized in this experimental model.

The cooling system failure and the consequent massive heat stroke mortality in all the experimental groups terminated some experimental components ahead of time (e.g., egg production monitoring), although the major monitored parameter, the post challenge mortality, seems not to be affected. The post challenge mortality ceased completely 3 days before the heat
stroke incident. The culture and PCR results on samples collected a week post the heat stroke (Table 3.1; 3 weeks post challenge) indicted an early infection with MS in the *E. coli* group. All the 3 post challenge mortalities in the *E. coli* group were MS negative by culture. We assume that the MS introduction into this group occurred during the heat stroke turmoil, and did not affect the pre heat stroke results. The PFGE patterns similarity between the *E. coli* isolated from the one mortality in the negative control group (Table 3.2) and the avian *E. coli* V9 challenge strain was unexpected. Possible explanations could be mislabeling, laboratory error or carryover from one of the *E. coli* challenge groups.

In summary, this study offers a useful challenge model for the reproduction of layer *E. coli* peritonitis syndrome. The results imply a possible pathogenesis mechanism for the disease which is in agreement with the clinical observations of respiratory origin of the layer *E. coli* peritonitis syndrome. The general notion of colibacillosis as a secondary disease was also demonstrated for the layer *E. coli* peritonitis syndrome, and a virulent MS strain appeared to be a possible primary factor in this syndrome.

**AKNOWLEDGMENTS**

We thank Dr. Richard E. Wooley for providing the *E. coli* V9 strain, Dr. John Maurer for performing the *E. coli* PFGE analysis, Dr. Susan Williams for the histopathology assistance, and Dr. Nicole Lazar for the help with statistics.
REFERENCES


Table 3.1. Summary of the MS monitoring results post the MS challenge.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Days post challenge</th>
<th>MS isolation&lt;sup&gt;B&lt;/sup&gt;</th>
<th>MS PCR&lt;sup&gt;A&lt;/sup&gt;</th>
<th>MS SPA</th>
<th>MS HI</th>
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</thead>
<tbody>
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<td>0/16</td>
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<td></td>
<td>21</td>
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<td>0/16</td>
<td>0/16</td>
</tr>
<tr>
<td></td>
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<td>0/10</td>
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<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
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<td>0</td>
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<td>0/5</td>
<td>0/16</td>
<td>0/16</td>
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<tr>
<td></td>
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<td>4/15</td>
<td>1/5</td>
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<td>0/15</td>
</tr>
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<td></td>
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<td>7/10</td>
<td>3/3</td>
<td>4/10</td>
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<tr>
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<td></td>
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<td>3/3</td>
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<td>10/10</td>
<td>3/3</td>
<td>10/10</td>
<td>6/10</td>
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</tbody>
</table>

<sup>A</sup> Pools of 3 or 4 tracheal swabs.

<sup>B</sup> Culture was positive by immunofluorescence for MS only.

<sup>C</sup> Only 15 birds survived the heat stroke in this group (see the mortality analysis section of the results for details).
Table 3.2. Post avian *E. coli* challenge daily mortality in the 4 experimental groups.

<table>
<thead>
<tr>
<th>Days post challenge</th>
<th>Neg. Ctrl</th>
<th><em>E. coli</em></th>
<th>MS</th>
<th>MS+<em>E. coli</em></th>
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</thead>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11&lt;sup&gt;A&lt;/sup&gt;</td>
<td>42</td>
<td>45</td>
<td>47</td>
<td>33</td>
</tr>
</tbody>
</table>

<sup>A</sup> heat stroke due to the experimental facility cooler failure (see the mortality analysis section of the results for details).
Table 3.3. Summary of the 10 days post avian *E. coli* challenge mortality.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Number of dead birds</th>
<th>Percentage of dead birds</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>1</td>
<td>1.56</td>
<td>B</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3</td>
<td>4.68</td>
<td>A B</td>
</tr>
<tr>
<td>MS</td>
<td>0</td>
<td>0</td>
<td>B</td>
</tr>
<tr>
<td>MS + <em>E. coli</em></td>
<td>5</td>
<td>7.81</td>
<td>A</td>
</tr>
</tbody>
</table>

^A Chi-square test for equality of proportions.
Fig. 3.1. Molecular typing of avian *Escherichia coli* by Pulsed Field Gel Electrophoresis (PFGE) using restriction enzyme, *Xba* I. Lanes 1 and 15: Lambda DNA Ladder (BioRad; Hercules, CA); lanes 2, 8 and 14: *E. coli* K12 control; Lane 13: PDRC clinical *E. coli* isolate as control; lane 3: *E. coli* strain V9; lanes 4-7 and 9-11 *E. coli* isolated from birds challenged with *E. coli* strain V9; Lane 12: *E. coli* isolated from the one mortality in the negative control group.
Table 3.4. Summary of tracheal mucosal thickness analysis and body cavity lesions results detected 4 weeks post challenge.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Tracheal mucosal thickness</th>
<th>Body cavity lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (μm)</td>
<td>Statistical significance (P ≤ 0.05)</td>
</tr>
<tr>
<td>Neg. Ctrl</td>
<td>70.24</td>
<td>B</td>
</tr>
<tr>
<td>E. coli</td>
<td>117.13</td>
<td>A B</td>
</tr>
<tr>
<td>MS</td>
<td>151.61</td>
<td>A</td>
</tr>
<tr>
<td>MS plus E. coli</td>
<td>165.72</td>
<td>A</td>
</tr>
</tbody>
</table>

A Mean of 10 tracheas mucosal thickness measured at four equidistant points.

B Tukey–Kramer HSD test.
Chi-square test for equality of proportions.
CHAPTER 4

THE MYCOPLASMA GALLISEPTICUM 16S-23S tRNA INTERGENIC SPACER REGION SEQUENCE, AS A NOVEL TOOL FOR EPIZOOTIOLOGICAL STUDIES

The *Mycoplasma gallisepticum* 16S-23S rRNA Intergenic Spacer Region Sequence, as a Novel Tool for Epizootiological Studies

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**ABSTRACT.** *Mycoplasma gallisepticum* (MG) contains two sets of rRNA genes (5S, 16S and 23S) in its genome, but only one of the two is organized in an operon cluster and contains a unique 660 nucleotide intergenic spacer region (IGSR) between the 16S and the 23S rRNA genes. We designed a polymerase chain reaction (PCR) for the specific amplification of the complete MG IGSR segment. The MG IGSR PCR was tested on 18 avian mollicute species and was confirmed MG specific. The reaction sensitivity was demonstrated while comparing it to the well established MG *mgc2* PCR. The MG IGSR sequence was found to be highly variable (discrimination (*D*) index of 0.950) among a variety of MG laboratory strains, vaccine strains, and field isolates. The sequencing of the MG IGSR appears to be a valuable single-locus sequence typing (SLST) tool for MG isolate differentiation in diagnostic cases and epizootiological studies.
Key words: *Mycoplasma gallisepticum*, 16S rRNA, 23S rRNA, Intergenic Spacer Region, Polymerase Chain Reaction, isolate, strain, Single-Locus Sequence Typing.

Abbreviations: AFLP = Amplified Fragment Length Polymorphism; bps = base pairs; GTS = Gene-Targeted Sequencing; IGSR = Intergenic Spacer Region; MG = *Mycoplasma gallisepticum*; MLST = multilocus sequence typing; PCR = Polymerase Chain Reaction; PFGE = Pulsed-Field Gel Electrophoresis; RAPD = Random Amplified Polymorphic DNA; RFLP = restriction fragment length polymorphism; SLST = single-locus sequence typing
INTRODUCTION

*Mycoplasma gallisepticum* (MG) is the most pathogenic and economically significant mycoplasma pathogen of poultry. Economic losses from condemnation or downgrading of carcasses, reduced feed and egg production efficiency, and increased medication costs are additional factors that make this one of the costliest disease problems confronting commercial poultry production worldwide (20). Isolates of MG are known by their isolate’s laboratory code or other designations, and could be referred to as strains. MG strains, including well-established reference strains, may differ markedly in their antigen profiles and their virulence-related surface properties (27). It has become increasingly important to develop methods to characterize and identify MG strains and strain variability. Reliable methods for the differentiation of MG strains play a pivotal role in understanding the epizootiology and spread of the disease because they generate the information necessary to identify and track new outbreaks. Also, the increased use of live MG vaccines requires more powerful tools to trace the source of infection and to differentiate vaccine strains from circulating field isolates. Recognition of intraspecific (strain) genotypic and phenotypic heterogeneity may be done by serologic methods (15, 27) or electrophoretic analysis of cell proteins (13). However, molecular techniques can be more sensitive and discriminatory for the differentiation of MG strains (21). Several techniques have been developed, including, restriction fragment length polymorphism (RFLP) (15), ribotyping (30), strain-specific DNA probes (14), pulsed-field gel electrophoresis (PFGE) (24), PCR with strain-specific primers (25), amplified fragment length polymorphism (AFLP) (10), and the currently most widely used random amplified polymorphic DNA (RAPD) (2, 5).

Sequencing methods have been introduced as a new approach for studying the molecular epidemiology of bacterial pathogens (1). Multilocus sequence typing (MLST) of housekeeping
genes has been demonstrated to be a highly transferable typing method, readily applicable to a wide variety of bacteria, which has contributed to the understanding of global epidemiology and population structure of infectious diseases (22). Also the sequencing of a single variable chromosomal locus (single-locus sequence typing (SLST)) was demonstrated as a promising approach for the detection of bacterial strain variation (17). Progress in the molecular biology of MG and the availability of the complete genome sequence (26) has driven the idea to evaluate chromosomal loci sequencing as a typing tool for differentiating MG strains. It was demonstrated recently that gene-targeted sequencing (GTS) analysis of MG surface-protein genes is a reproducible typing method with satisfactory discriminatory power to separate isolates from unrelated outbreaks, and to identify closely related isolates (3). Unlike other widely used MG typing methods (e.g., RAPD), the sequencing methods do not require the isolation of the tested organism in a pure culture. Mixed infection with MG and saprophytic mycoplasmas is a common occurrence in poultry flocks (23), and the independence of the sequencing methods on the recovery of MG in a pure culture is a significant advantage.

The members of the class *Mollicutes* have only one or two copies of the rRNA genes. Some of the genes coding for rRNA molecules of the genus *Mycoplasma* are organized in operons and arranged in the order 5’ 16S-23S-5S 3’ in which the individual rRNA genes are separated by internal transcribed spacer regions. The intergenic spacer region (IGSR) between the 16S and 23S rRNA genes of mycoplasmas has been shown to lack tRNA genes and to be variable in sequence and length among mycoplasma species (29). It has been used as a genetic marker for comparing phylogenetic relationships of genetically closely related species among not only the mycoplasmas (8, 28), but also other bacterial species (6, 7, 12, 19). MG contains two sets of rRNA genes (5S, 16S, and 23S) in its genome, but only one of the two is organized in an
operon cluster and contains a unique 660 nucleotide IGSR between the 16S and the 23S rRNA genes (26).

This is the first report on the implementation of the SLST approach for MG strain differentiation. Our objectives were to determine the level of the intraspecific genotypic polymorphism of the MG 16S-23S rRNA IGSR (MG IGSR), and to evaluate the discriminatory power of this single chromosomal locus for MG isolates in diagnostic cases and epizootiological studies.

**MATERIALS AND METHODS**

**Mollicute species and Mycoplasma gallisepticum strains and isolates.** Eighteen different avian mollicute species (Table 4.1) from our laboratory archive were used to demonstrate the MG IGSR PCR specificity. A variety of MG strains and isolates: 3 MG GenBank sequences, 6 MG laboratory strains, 3 MG commercial vaccine strains, and 26 MG field isolates from unrelated US cases, were used to evaluate the intraspecific variability level of the MG IGSR sequence (Table 4.1).

**DNA extraction.** DNA was extracted from cultures grown in modified Frey’s broth at 37°C or frozen modified Frey’s medium stock cultures stored with 5% (v/v) glycerol (16). Genomic DNA was extracted from 200 μl of mycoplasma culture in modified Frey’s broth or from 200 μl of tracheal swab pool dipped in PBS using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) following the manufacturer’s recommendations.

**PCR amplification and sequencing of the complete MG 16S-23S rRNA IGSR.** Primers were designed with the PrimerSelect program (in Lasergene; DNASTAR, Inc., Madison, WI). The primers’ annealing sites were designed in the downstream region of the 16S rRNA
gene and the up stream region of the 23S rRNA gene for the complete amplification of the MG IGSR segment. The primers’ sequence, location, and expected PCR product size of the MG IGSR PCR, based on the MG R (low) genome sequence (GenBank AE015450), are presented in Table 4.2. A BLAST search was performed online (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm the theoretical specificity of the primers. The reaction mix was purchased from EPICENTRE (EPICENTRE Biotechnologies, Madison, WI), and contained 25µl of FailSafe PCR 2X PreMix B, 0.1µM primers, 0.5µl FailSafe PCR Enzyme Mix, 5µl DNA template, and water to a volume of 50µl. Amplifications were performed in a PTC-200 DNA Engine MJ thermocycler (MJ Research) at 94°C for 3 min, 30 cycles of 94°C for 20 sec, 55°C for 30 sec, 72°C for 60 sec, and ending with 72°C for 5 min.. The PCR products were separated on a 2% agarose gel containing 1 mg/ml ethidium bromide and visualized by UV transillumination. The amplification products were then purified using a QIA PCR purification kit (QIAGEN Inc., Valencia, CA) and sent to the Integrated Biotechnology Laboratory (University of Georgia, Athens) for sequencing. Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit and the capillary sequencer 3100 ABI Genetic Analyzer (Applied Biosystems, Foster City, CA). Each amplification product was sequenced in both directions with the forward and reverse amplification primers. Raw sequence data was analyzed with the EditSeq program (in Lasergene; DNASTAR, Inc., Madison, WI), and the complete overlapping of complementary sequences, editing and consensus construction were produced with the SeqMan program (in Lasergene; DNASTAR, Inc., Madison, WI).

**PCR amplification of the MG mge2.** To evaluate the MG IGSR PCR sensitivity it was compared to our laboratory standard MG (mge2) PCR. The reaction was performed following the
protocol published by Garcia et al. (4). The PCR products were separated on a 2% agarose gel containing 1 mg/ml ethidium bromide and visualized by UV transillumination.

**SLST analysis of the MG 16S-23S rRNA IGSR.** To conduct SLST analysis all consensus sequences were edited to start at an equivalent coding sequence position using the EditSeq program (in Lasergene; DNASTAR, Inc., Madison, WI). Alignments of sequences were constructed by the Clustal V method with a gap penalty of 10 using the MegAlign program (in Lasergene; DNASTAR, Inc., Madison, WI). Dendrograms were constructed from the Clustal V alignments by the neighbor joining method and 1000 bootstrap replicate analysis using the MOLECULAR EVOLUTIONARY GENETIC ANALYSIS (MEGA) software available at www.megasoftware.net for sequence alignments (18).

**Sequence stability of the MG 16S-23S rRNA IGSR.** In order to evaluate the stability of the targeted region, amplification and sequencing were performed on the laboratory strains R (low) and S6, and on the vaccine strains ts-11 and 6/85 before and after several in vitro passages (Table 4.1).

**Tracheal swabs sampling.** Sterile cotton-tipped applicators were utilized to swab the trachea of 6-wk-old specific-pathogen-free leghorn chickens 10 days post MG R (low) challenge. Tracheal swabs from three consecutive birds were pooled in 1ml PBS for PCR analysis.

**Discrimination index.** The overall discriminatory power of a typing method is defined as its ability to distinguish between different strains. This can be expressed as an index that measures the probability that two unrelated strains will be placed into different groups, and is calculated using Simpson’s diversity or discrimination ($D$) index, which takes into account the number of types defined by the method and the relative frequencies of these types (11). A $D$ index of $>0.90$ is considered adequate, and a $D$ index $>0.95$ is considered as a good typing
discrimination power. Isolates with identical sequences (100% identity) were considered as the same sequence type. This index can be derived from elementary probability theory and is given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j-1)$$

Where N is the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the jth type.

RESULTS

MG 16S-23S rRNA IGSR PCR specificity assay. The designed PCR was performed on the eighteen different avian mollicute species listed in Table 4.1, including MG as a positive control, to verify the reaction specificity. The reaction was negative for all the species in Table 4.1, except from Mycoplasma imitans that yielded a band of higher molecular weight (~2500bps) than the MG control (~800bps).

MG 16S-23S rRNA IGSR PCR sensitivity assay. DNA was extracted from two pools of three tracheal swabs collected from birds 10 days post MG R (low) strain challenge, and from one MG R (low) strain culture. Ten-fold serial dilutions down to $10^{-4}$ were prepared from the above three DNA extracts. The MG IGSR PCR and the MG mgc2 PCR reactions were performed on these three dilution sets, and run on the same gel. The results (Fig. 4.1) indicated a better sensitivity of the MG IGSR PCR, and also demonstrated inconsistent amplification result for the MG mgc2 PCR versus highly consistent amplification result for the MG IGSR PCR.

MG 16S-23S rRNA IGSR sequencing results. The thirty-seven unrelated strains/isolates in Table 4.1 were sequenced and aligned. The results are presented in the form of
a phylogenetic dendogram (Fig. 4.2). The strains/isolates were grouped into 22 different types: one type with 7 strains/isolates (6/85 like), 3 types with 3 strains/isolates, 3 types of 2 strains/isolates, and 15 types of one strain/isolate.

**MG 16S-23S rRNA IGSR sequence stability.** The MG IGSR sequence stability was evaluated by sequencing several *in vitro* passages of the laboratory strains R (low) and S6, and the vaccine strains ts-11 and 6/85. Sequences of R (low) strain from GeneBank (AE015450), following 105 passages and following 120 passages were all 100% identical. Likewise, sequences of strain S6 at passage levels 15, 21 and 29 were 100% identical. The sequences of the two vaccine strains, ts-11 and 6/85, were also perfectly stable following 0 (commercial vaccine bottle), 126, and 141 passages.

**MG 16S-23S rRNA IGSR sequence discrimination index.** The sequence analysis of the 37 unrelated MG strains and isolates identified 22 sequence types with a discrimination \((D)\) index of 0.95045.

**DISCUSSION**

This study describes the first utilization of the SLST approach for differentiation of MG isolates. The possibility of using a single variable chromosomal locus with high discriminatory power for differentiation of MG isolates harbors significant advantages over the currently used methods. The freedom from the requirement to culture the organism, the 100% typeability and reproducibility, the quick accumulation of reference data base, and the ease and rapid application are the major advantages of a single locus sequencing method. Another important advantage is the ability to perform the SLST analysis as part of the diagnostic MG PCR test. The main disadvantage of the SLST approach is the analysis of a very limited portion of the genome, and
when differences are not detected it is required to continue and search for differences with alternative methods before a conclusion can be reached.

The rRNA IGSR size of most avian mycoplasmas is smaller than five hundred base pairs (bps). Exceptions to this rule are MG and *Mycoplasma imitans*, which contain IGSRs of 660 bps and 2488 bps, respectively (9). Albeit that in several mollicutes very low intraspecific variability of the rRNA IGSR has been reported (31), we hypothesized that the unique MG IGSR is a reasonable place to look for intraspecific genotypic polymorphism. We assumed that a relatively large nonsense genome segment with no reading frame constraints, and with no homologous counterpart sequences in the genome to allow gene conversion repair, could harbor evolutionary accumulation of genotypic changes. We also based our hypothesis on the knowledge that in other prokaryotes intraspecific variations in the lengths and sequences of rRNA IGSR have been described (6, 7, 12, 19).

The results indicated significant sequence variability and good discriminatory power among MG strains by the MG IGSR sequence. The discriminatory \(D\) index of 0.950 was found to be superior to the previously analyzed MG genes: *gapA*, *MGA_0319*, *mgc2* and *pvpA* with discriminatory indices of 0.713, 0.874, 0.915 and 0.920, respectively (3). The 37 strains and isolates that we used to sequence the MG IGSR were also used by Ferguson et al. (3). These 37 strains/isolates were grouped into ten different genotypes by the four genes GTS array (*gapA*, *MGA_0319*, *mgc2* and *pvpA*) and RAPD in Ferguson’s study. The MG IGSR sequence could further differentiate these ten genotypes into fourteen distinct genotypes. It appears that the MG IGSR sequence has a promising potential as an independent SLST tool for MG isolates, and could serve as an important addition to the described (3) multiple MG GTS method.
Sequence stability of the MG IGSR was demonstrated through numerous *in vitro* passages. The region’s sequence stability, and our accumulating experience in comparing MG IGSR sequences with other genotyping methods (unpublished), led us to conclude that even one base variation is enough indication for isolate differentiation. Another important observation was that none of the study’s strains and field isolates shared the ts-11 vaccine strain MG IGSR sequence. This finding is important in the context that according to our laboratory data several field isolates share the ts-11 *mgc2* sequence (unpublished); the *mgc2* gene PCR is widely used in this laboratory for diagnostic purposes. This superior differentiating capability of the MG IGSR versus the *mgc2* gene could be useful in situations where the involvement of the ts-11 vaccine strain is suspected. This MG IGSR characteristic could address the increased need of the poultry industry for non-culture dependent differentiating tools between vaccine strains and field isolates.

The MG IGSR PCR was tested on samples from live birds and cultures under controlled comparison conditions, and appeared to be more sensitive than the commonly used MG *mgc2* PCR. The reaction was also highly specific; the amplification of the *M. imitans* rRNA IGSR was expected since these two closely related mycoplasmas having nearly identical rRNA sequences. The size difference between the MG amplicon and the *M. imitans* amplicon is due to a putative transposase insertion in the *M. imitans* 16S-23S rRNA IGSR (9), and makes these two species distinguishable by the MG IGSR PCR reaction.

In summary, the uniquely sized MG 16S-23S rRNA IGSR sequence harbored significant genotypic polymorphism that could be utilized as a preliminary (SLTS) or complementary tool in MG strain differentiation in diagnostic cases and epizootiological studies. The MG IGSR PCR
demonstrated high sensitivity and specificity and could be a feasible alternative to the currently available MG PCRs.

REFERENCES


Table 4.1. Avian Mollicute species and *Mycoplasma gallisepticum* strains and isolates used in this study:

<table>
<thead>
<tr>
<th>Organism source</th>
<th>Species, strains and isolates</th>
</tr>
</thead>
</table>
| Avian Mollicute species: | *Acholeplasma laidlawii, Mycoplasma anatis, Mycoplasma cloacae,*  
| | *Mycoplasma columbinasale, Mycoplasma columbinum, Mycoplasma columborale, Mycoplasma gallinarum, Mycoplasma gallinaceum,*  
| | *Mycoplasma gallopavonis, Mycoplasma glycophilum, Mycoplasma iners,*  
| | *Mycoplasma iowae, Mycoplasma lipofaciens, Mycoplasma meleagridis,*  
| | *Mycoplasma pullorum, Mycoplasma synoviae, Mycoplasma imitans,*  
| | *Mycoplasma gallisepticum* |
| GenBank sequence accession # of MG strains: | PG31 AB098504, A5969 L08897, R (low) AE015450 |
| MG laboratory strains: | A5969 Kleven, A5969 Geary, A5969 liposome, S6(15 passages), S6(21 passages), S6(29 passages), R low (105 passages), R low (120 passages), HF-51 |
| MG vaccine strains: | F, 6/85(0 passages), 6/85(126 passages), 6/85(141 passages), ts-11(0 passages), ts-11(126 passages), ts-11(141 passages) |
| MG unrelated US isolates*: | K2101CK84, K4029/1TK95, K4110BTK96, K4158CTK96, K4181BCK96, K4181CCK96, K4236/3TK96, K4246TK96, K4280CK96, K4385TK97, K4421A/1TK97, K4423BTK97, K4465/2TK97, K4649BTK98, K4688FCK98, K4781ATK97, K4902ETK00, K5011TK00, K5037ACK00, K5054TK01, K5058ETK01, K5234/5CK02, K5263E/10CK02, K5792A/5CK05, K5792B/2CK05, K5833/1TK05 |

*each isolate K number is followed by the species abbreviation and the year of isolation.*
Table 4.2. MG IGSR PCR primers’ sequence, location, and expected PCR product size, based on the MG R (low) genome sequence (GenBank AE015450).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→ 3’)</th>
<th>Genome nt position</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG IGSR F</td>
<td>GTAGGGCCGGTGATTGGAGTTA</td>
<td>321490- 321511</td>
<td>812bps</td>
</tr>
<tr>
<td>MG IGSR R</td>
<td>CCCGTAGCATTTCGCAGGTGG</td>
<td>322301- 322280</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4.1. Sensitivity analysis of MG IGSR PCR vs. mgc2 PCR. The upper row was used for the mgc2 PCR products, and the lower row was used for the MG IGSR PCR products. Lanes 1 and 25: 100bps ladder. Lanes 9 and 17 are negative controls. Lanes 3 to 7 are ten-fold dilutions from undiluted down to $10^{-4}$, respectively, of pool one prepared from three tracheal swabs collected from post challenge birds. Lanes 11 to 15 are ten-fold dilutions from undiluted down to $10^{-4}$, respectively, of pool two prepared from three tracheal swabs collected from post challenge birds. Lanes 19 to 23 are ten-fold dilutions from undiluted down to $10^{-4}$, respectively, prepared from MG R (low) strain culture.
Fig. 4.2. Dendrogram constructed with MG IGSR sequences from 37 unrelated MG strains and isolates using the neighbor joining (NJ) method with 1000 bootstrap replicates using MEGA3.1 (www.megasoftware.net). The numbers denote the confidence interval percentage for a certain branch to occur. GenBank, laboratory strains, and vaccine strains sequences are in bold italic. Each US field isolate K number is followed by the species abbreviation and the year of isolation.
CHAPTER 5

INTRASPECIFIC DIFFERENTIATION REAL-TIME PCR FOR *MYCOPLASMA GALLISEPTICUM* LIVE VACCINE EVALUATION

3 Raviv, Z., Callison, S., Ferguson, N., Kleven, S. H. To be submitted to Infection and Immunity.
Intraspecific Differentiating Real-Time PCR for \textit{Mycoplasma gallisepticum} Live Vaccine Evaluation

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\textbf{ABSTRACT.} \textit{Mycoplasma gallisepticum} causes respiratory disease and production losses in poultry. Vaccination of poultry with \textit{Mycoplasma gallisepticum} live vaccines is an efficacious approach to reduce susceptibility to infection and to prevent the economic losses. The development and evaluation of live vaccines usually requires the involvement of several vaccine and challenge strains in the same experimental setup. Our goal was to develop a tool to allow the absolute differentiation between a set of known \textit{Mycoplasma gallisepticum} strains in a quantitative manner. We developed 5 real-time PCR assays that absolutely differentiated between one of the five commercial and laboratory vaccine strains: F, ts-11, 6/85, K5831, K5054, and the standard challenge strain $R_{\text{low}}$ when tested on \textit{in vitro} cultures. The assay K5831 vs. $R_{\text{low}}$ was also tested on specimens from live birds that were vaccinated with K5831 and challenged with $R_{\text{low}}$, and successfully differentiated between the vaccine and the challenge strains in a quantitative manner. This preliminary \textit{in vivo} application of the method also shed light on possible protection mechanisms for the \textit{Mycoplasma gallisepticum} K5831 vaccine strain.
Key words: *Mycoplasma gallisepticum*, live MG vaccine, MG strain, real-time PCR, dual-labeled probe.

Abbreviations: AFLP = amplified fragment length polymorphism; CCU = color changing units; C_T = threshold cycle; HI = hemagglutination inhibition; MG = *Mycoplasma gallisepticum*; PCR = polymerase chain reaction; PBS = phosphate buffer saline; SPA = serum plate agglutination. RAPD = random amplified polymorphic DNA.
INTRODUCTION

*Mycoplasma gallisepticum* (MG) is an infectious respiratory pathogen of chickens and turkeys. When present in concert with other respiratory pathogens, such as infectious bronchitis virus, Newcastle disease virus, *Escherichia coli*, or *Haemophilus paragallinarum*, a condition known as chronic respiratory disease results. The disease symptoms are characterized by rales, coughing, nasal discharge, and conjunctivitis, with infraorbital sinusitis frequently seen in turkeys (16).

*Mycoplasma gallisepticum* is the most pathogenic and economically significant mycoplasma pathogen of poultry. Economic losses from condemnation or downgrading of carcasses, reduced feed and egg production efficiency, and increased medication costs are factors that make this one of the costliest disease problems confronting commercial poultry production worldwide. Prevention and control programs account for additional costs (16).

Transmission of MG can occur through the egg or by inhalation of contaminated airborne droplets, resulting in rapid disease transmission throughout the flock (16). Traditional prevention and control programs were based on strict biosecurity, surveillance (serology, culture, and molecular identification), and eradication of infected breeder flocks. The rapid expansion of poultry production in restricted geographical areas and the consequent recurring MG outbreaks necessitated the implementation of additional measurements.

Vaccination with bacterins has been shown to reduce, but usually not eliminate, colonization by MG following challenge. Generally, it is felt that bacterins are of minimal value in long-term control of infection on multiple-age production sites (16). Live MG vaccines appear to be more effective, and therefore more popular, than bacterins (11, 20). Vaccination with live MG vaccines has become an accepted management tool for the control of MG in chickens and in
some situations it is the preferred control option, particularly when multiple age flocks are housed on the same site (3). An important characteristic of MG live vaccines is their ability to induce resistance to wild-type strain infection, and to displace wild-type strains with the vaccine strain on multiple-age production sites (15). Currently there are 3 commercially licensed vaccines, containing living cultures of either F (1), 6/85 (4) or ts-11 (19) strains of MG. The available MG live vaccines have shown little potential for use in turkeys (16).

Live vaccine development and evaluation require studies that involve two or more MG strains in the same experimental setup. Protection study formats can include only one vaccine strain and one challenge strain (17), or a few vaccine strains in different experimental groups challenged by the same virulent strain (6). Displacement studies, to evaluate the capability of vaccine strains to displace a virulent strain, utilize several vaccine strains and a challenge strain (12). The study of the immune mechanisms by which the MG vaccines confer protection from challenge also requires the involvement of at least two strains (9). In all the different MG live-vaccine evaluation study formats, the involved strains could not be well differentiated and analyzed separately from one another once they were introduced into the experimental system. This lack of ability to differentiate between the participating strains limits the level of control and the amount of information that could be gained from MG vaccine evaluation studies. Our objectives were to develop a research tool to allow the qualitative and quantitative differentiation between MG strains utilized in vaccine evaluation studies and to improve the reliability and efficiency of these studies. In real-time PCR, the production of specific PCR products is monitored by fluorimetric detection of amplified products and identified by melting curve analysis or specific hybridization probes (21, 22). The reaction has an inherent quantitative nature, and benefits the convenience of a one step procedure. A real-time PCR with a dual-
labeled probe (Taqman) appears advantageous for microorganisms strain differentiation due to the superior sensitivity and improved specificity endowed by 3 hybridizing oligo nucleotides (two primers and a probe).

In this study, we present the concept of quantitative strain differentiating real-time PCR for the differentiation between MG strains in live vaccine evaluation studies. Based on known sequences of several genes from the 3 commercial MG vaccines (F, ts-11, 6/85), 2 laboratory vaccines (K5831, K5054), and the common challenge strain R_{low}, 5 assays (each vaccine vs. R_{low}) were designed. The 5 assays absolutely differentiated between in vitro cultures of the designated vaccine strain and the R_{low} challenge strain. The implementation of this novel tool in an in vivo MG vaccine protection study revealed an interesting insight into the quantitative relationships between the vaccine and the challenge strains in the chicken trachea and to a putative protection mechanism induced by the vaccine strain.

**MATERIALS AND METHODS**

**MG strains and target gene sequences.** Five MG live vaccine strains and one challenge strain were used in this study. The three MG commercial vaccine strains: F (Schering-Plough, Summit NJ), ts-11 (Merial Select, Gainesville, GA) and 6/85 (Intervet America, Millsboro, DE) were used directly from sealed containers supplied by the manufacturer. The K5831 isolate from our laboratory depository was isolated in 2005 from chickens challenged with K2101. K2101 was isolated in 1984 from a commercial layer flock in Colorado, US, with drops in egg production. This isolate was used as an autogenous vaccine on the same farm with satisfactory results (Dr. S. H. Kleven, unpublished). The K5054 isolate from our laboratory depository was obtained from sinus exudate of commercial turkey breeders from Indiana in 2001 (5). Further
characterization of this isolate demonstrated its prophylactic properties (6). The MG \( R_{low} \) strain from our laboratory depository served as the challenge strain. \( R_{low} \) strain is a virulent MG strain, which has been previously described (18). The sequences of the \( mgc2 \), MGA_0319, and \( pvpA \) genes of the above 6 MG strains were published by Ferguson \textit{et al.} (7).

**DNA extraction.** DNA was extracted from commercial vaccine resuspensions in PBS, cultures grown in modified Frey’s broth at 37° C (13), tracheal swabs dipped in 1 ml PBS, or 1 ml laryngeal wash of vaccinated and challenged birds. Genomic DNA was extracted from 200 µl of the above solutions using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s recommendations.

**MG strain differentiating real-time PCR primers and probes.** Five real-time PCR assays were designed based on the known sequences of the \( mgc2 \), MGA_0319, and \( pvpA \) genes (GenBank accession numbers listed in Table 5.1, a-e). Each assay included 2 separate Taqman real-time PCRs, one specific for the vaccine strain and the other specific for the \( R_{low} \) challenge strain. All primers and a dual-labeled probes (TaqMan) were supplied by Integrated DNA Technologies (IDT Inc., Coralville, IA). The primer and probe sequences and their location on the GenBank sequences are presented in Table 5.1, a-e. The K5831 and K5054 strains share identical sequence on the amplified region of the \( mgc2 \) gene and the same reaction was utilized for both (Table 5.1, a-b).

**MG strain differentiating real-time PCR protocol.** Primers and probes were utilized in a 25 µl reaction mix containing 12.5 µl of Quantitect Probe PCR 2X mix (Qiagen, Valencia, CA), primers to a final concentration of 0.5 µM, and a probe to a final concentration of 0.1 µM, 3 µl of water, and 5 µl of template. Each reaction was performed in a SmartCycler (Cepheid, Sunnyvale, CA) using a thermocycle program of 95° C for 15 min with optics off; and 40 cycles
of 94° C for 15 sec followed by the reaction specific annealing and extension temperatures as specified (Table 5.1, a-e), for 60 seconds with optics on. The threshold cycle number (C_{T} Value) was determined to be the PCR cycle number at which the fluorescence of the reaction exceeded 30 units of fluorescence. Any reaction that had a recorded C_{T} value was considered positive and any reaction that had no recorded C_{T} value was considered negative.

**Preparation of standard DNA controls.** The *mge2* gene of the 5 MG vaccine strains used in this study, and the MGA_0319 and *pvpA* genes of the MG R_{low} strain were amplified by PCR as previously described (7). Each amplicon (K5831 and K5054 identical) was gel-purified and cloned into the pCR®2.1-TOPO® vector and transformed into One Shot® TOP10 competent *E. coli* cells using the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) following the manufacturer’s recommendations. From each of the transformation assays, colonies (white colonies) were selected and grown up in LB broth with 50 μg/ml Kanamycin. Plasmid DNA was extracted using a DNA MiniPrep kit (Qiagen, Valencia, CA) per the manufacturer’s recommendations. Clones containing the proper gene insert were verified by sequencing (utilizing the vector primers M13F and M13R). A sequence-verified plasmid from each of the transformant cultures was purified and used to construct a standard curve and to determine the quantitation limit for each of the developed reactions. Validated plasmids were stored at -70° C as standard DNA controls.

**Standard curves.** The quantitation and detection limit of each of the study’s real-time PCRs were determined by 3 independent runs of each reaction, using 10-fold serial dilutions (10^8 to 10^1 copies per reaction) of the reaction target gene cloned into plasmid DNA as template. Each reaction standard curve was generated by plotting the 3 run mean C_{T} values vs. \log_{10} of the plasmid template copy numbers.
Specificity test. Each of the study’s differentiating assays was performed on genomic DNA extracts from stationary phase cultures of the assay’s pair of strains.

In vivo study and sample collection. Ninety-six 8 weeks old mycoplasma free commercial layer type chickens were allotted into 8 groups of 12 chickens each. Six groups were vaccinated via aerosol with ten-fold dilutions of an MG K5831 culture ranging from $6.22 \times 10^1$ CCU/ml to $6.22 \times 10^6$ CCU/ml, with approximately 1 ml of diluted culture sprayed per bird. At 33 days post vaccination, all the chickens in all the experimental groups were sampled individually and tested by mycoplasma culture, serology, and real-time PCR. At 42 days post vaccination the 6 vaccinated groups and one non-vaccinated group were challenged with the MG $R_{low}$ strain in a dose of $2.51 \times 10^8$ CCU/bird by aerosol. One group was non-vaccinated/ non-challenged and used as negative control. Ten birds from each of the experimental groups were necropsied at 10 days after the $R_{low}$ strain challenge, analyzed individually for air sac lesions and sampled by mycoplasma culture and serology. Six necropsied birds per group were sampled for real-time PCR by laryngeal wash; the larynx was cut exactly at the base (for uniformity purposes), put in 10 ml sterile plastic tubes filled with 4 ml PBS, and vortexed for 30 seconds. A 1 ml laryngeal wash solution was submitted for DNA extraction. Tracheal sections were submitted for tracheal mucosal width analysis as described below.

Mycoplasma isolation and identification. Choanal cleft swabs collected from the in vivo study birds were submitted for mycoplasma culture. They were inoculated in Frey’s modified broth and agar and incubated at 37°C. Mycoplasma isolates were identified by direct immunofluorescence (13).
MG serology. Serum samples from the *in-vivo* study were tested for MG by the serum plate agglutination assay (SPA) (Intervet, Millsboro, DE) according to the manufacturer’s instructions, and the hemagglutination inhibition test (HI) as described (13).

Evaluation of lesions. To evaluate the level of protection induced by the K5831 strain vaccination to the chickens’ upper respiratory tract, tracheal lesions were evaluated microscopically by measuring the width of the tracheal mucosa. A section of the upper third of the trachea (approximately 2 cm distal from the larynx) was fixed in 10% neutral buffered formalin. The tracheal mucosa thickness was measured at four equidistant points on histologic slides of cross sections of tracheas (20). The level of protection to the lower respiratory tract was evaluated by gross air sac lesion scoring on a scale from 0 to 4 (10).

Statistical analysis. The HI titers, tracheal mucosa thickness and K5831 vs. R_low assay results were analyzed by the Tukey–Kramer HSD test, and the SPA, MG isolation, and air sac lesion results were analyzed by the Kruskal-Wallis Rank Sums test (JMP_ Statistics Made Visual; SAS Institute, Inc., Cary, NC). A *P*-value of ≤0.05 was considered significant in all the analyzed tests.

RESULTS

Strain differentiating assays specificity. Each of the study’s 5 differentiating assays aim to differentiate between 2 MG strains (vaccine and challenge), and was constructed of 2 mutually exclusive strain reactions. The differentiating assays specificity was tested by performing each assay’s pair of Taqman real-time PCRs on *in vitro* cultures of the assay’s target strains. Each strain reaction was demonstrated to be highly specific and sensitive to the reaction’s target strain and absolutely negative to the reaction’s reciprocal strain in each particular assay. The results,
expressed as $C_T$ values, of the study’s 5 differentiating assay specificity tests are summarized in Table 5.2.

**Strain differentiating assays quantitation and detection limits.** Standard curves were assembled for the 6 Taqman real-time PCRs that construct the study’s 5 strain differentiating assays. Each reaction standard curve was determined by 3 independent runs of each reaction using 10-fold serial dilutions ($10^8$ to $10^1$ copies per reaction) of the reaction’s standard DNA control. The mean $C_T$ values, the linear equation and the R-squared value of the obtained standard curves are summarized in Table 5.3. Five out of the 6 reactions were highly sensitive and detected 10 copies of plasmid DNA per reaction (25 μl) within the linear phase of the standard curve. The MG $R_{low}$ strain ($pvpA$) reaction was adequately sensitive and detected 100 copies of plasmid DNA per reaction within the linear phase of the standard curve.

**The in vivo study results.** To evaluate the developed method in a live bird situation a pilot *in vivo* study was performed. Chickens were vaccinated with a series of ten-fold dilutions of the MG K5831 strain and 42 days later were challenged with the MG $R_{low}$ strain as described above. The experimental groups were sampled at 33 days post vaccination and at 10 days post challenge for the strain differentiating real-time PCR method, mycoplasma culture, and serology. The level of protection conferred to the vaccinated chicken respiratory tract was analyzed by the air sac lesion score and the tracheal mucosal width assay. The results of the post vaccination and post challenge tests are summarized in Tables 5.4 and 5.5, respectively. The post vaccination results indicated that only the 3 highest doses of the K5831 strain culture ($6.22 \times 10^4$, $6.22 \times 10^5$, $6.22 \times 10^6$ / CCU) were able to colonize the chicken tracheas and that all the experimental groups were $R_{low}$ strain negative. On the post challenge examination the birds that were vaccinated with the 2 highest doses of the K5831 strain, $6.22 \times 10^5$ / CCU and $6.22 \times 10^6$ /
CCU, demonstrated a sharp decrease in tracheal copy number of the R\textsubscript{low} challenge strain. These statistically significant lower tracheal copy numbers of the R\textsubscript{low} challenge strain coincided with the statistically significant lower level of tracheal and air sac lesion scores in these 2 experimental groups. Another interesting finding was the drop in copy number of both vaccine and challenge strains in the birds that were vaccinated with the highest dose of the K5831 strain (6.22 \times 10^6 / CCU).

**DISCUSSION**

The evaluation of live avian mycoplasma vaccines and the study of their mechanism of action have lacked the ability to differentially identify and quantify the participating strains within an experimental setup. The available DNA fingerprinting methods (e.g., RAPD, AFLP) are highly dependent on the isolation of the tested organisms in a pure culture (8), and are not suitable for multi strain infection situations. The lack of ability to differentially identify the participating strains imposed significant limitations to the level of control that could be achieved in live vaccine evaluation studies. After the introduction of the vaccine strain, it was no longer possible to accurately verify that only the vaccine strain was present in the experimental birds. In vaccinated bird challenge studies and displacement of field strains by vaccine strain studies, the inability to sensitively detect all the participating strains compromised the soundness of these studies’ conclusions. The mechanism of action of empirically protective vaccine strains could not be well studied without the ability to differentiate and quantify the involved strains in specimens from live birds. The development of the strain differentiating real-time PCR approach addresses the above issues in a highly sensitive, quantitative, and specific manner, as well as, provides investigators with a reliable and a feasible tool for live vaccine evaluation studies.
It is very important to clarify that the differentiation properties of the strain differentiating real-time PCR method is restricted to the known array of strains that were included in the designed assay. The design of this differentiation method was based on sequence differences in unique genomic regions between a known pair of strains. It is most likely that the targeted sequences that are absolutely different between the assay’s two strains are shared by other MG strains. Such other MG strains will not be differentiated from the assay’s target strain.

The purpose of the in vivo part of the study was to validate the performance of the developed method on samples from live birds, but it also provided an interesting insight into the live MG vaccine mode of action and protection mechanisms. The pre-challenge evaluation of the vaccine and the challenge strains confirmed that none of the experimental groups were prematurely exposed to the challenge strain (Table 5.4). The post-challenge quantitative evaluation indicated statistically significant lower copy numbers of the challenge strain in the groups that were successfully colonized by the K5831 vaccine (6.22 X 10^4, 6.22 X 10^5, 6.22 X 10^6 / CCU). Lower copy numbers of both the vaccine and the challenge strains were detected in the group vaccinated with the highest dose (Table 5.5). The lower copy numbers of the challenge strain in the higher dose vaccinated groups, 6.22 X 10^5 / CCU and 6.22 X 10^6 / CCU, correlated well with protection from tracheal and air sac lesions in these groups (Table 5.5). These observations could be meaningful for the understanding of the vaccine’s protective mechanisms. Suggestions that could be proposed and further evaluated include: the reduced copy number of the challenge strain is not due to competitive exclusion by the vaccine strain but the consequence of the immune response induced by the vaccine strain; the immune response induced by the non-virulent K5831 strain prevented the R_{low} challenge strain from massive tracheal colonization and effectively protected against tracheal and air sac lesions. It is interesting to note that two decades
ago quite a similar observation was made by Levisohn et al. (14) by utilizing drug resistance variation between MG strains for differentiation and agar plate colony count for quantitation. This preliminary data suggests possible protection mechanisms for the K5831 vaccine strain, and demonstrate the plentiful opportunities that the quantitative strain differentiating real-time PCR approach brings to the field of live mycoplasma vaccine research.

This pilot development of a quantitative strain differentiating real-time PCR approach focused on the method’s proof of concept. The real-time PCR with a dual labeled probe technology and the up-to-date real-time PCR equipment allow further improvements of the developed assays. One area for improvement could be the inclusion of the study’s dual reaction assays into a unified multiplex real-time PCR assay, as recently described in closely related viruses (2). Another possible improvement could be the inclusion of more than 2 strains per assay.

This is the first report of a molecular assay that can absolutely differentiate between a known array of intraspecific mycoplasma strains in a mixed sample. The real-time PCR with a dual labeled probe technology endowed the method with its superior sensitivity, specificity and quantitative properties. The initial application of this quantitative strain differentiating tool was designed for live mycoplasma vaccine evaluation studies and indeed provided a significant upgrade to this area of research. The demonstrated concept of intraspecific differentiating real-time PCR is general and could be considered for a variety of research applications in mycoplasmology and microbiology.
REFERENCES


**Table 5.1a.** The Taqman real-time PCR specifications of the K5831 (K2101) versus R<sub>low</sub> strain differentiating assay.

<table>
<thead>
<tr>
<th>Assay</th>
<th>K5831 (K2101)</th>
<th>R&lt;sub&gt;low&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene and GenBank seq. accession #</td>
<td>mgc2, AY556238</td>
<td>MGA_0319, AY556072</td>
</tr>
<tr>
<td>Forward (F) primer sequence (5'→3')</td>
<td>CTCAAGAACCAACTCAACCA</td>
<td>ACTAATAATACTAACCACCGATTAAC</td>
</tr>
<tr>
<td>Reverse (R) primer sequence (5'→3')</td>
<td>GGATGGACCAATATGCGGAT</td>
<td>GTAGTTCGATTCGTTTCACCTGTAT</td>
</tr>
<tr>
<td>Dual labeled probe (P) sequence (5'→3')</td>
<td>CAACCAGGATTTAATCAACCTCAG</td>
<td>AAATGGTAACACAGCCAACACCTACTC</td>
</tr>
<tr>
<td>PCR product size</td>
<td>112bps</td>
<td>170bps</td>
</tr>
<tr>
<td>Annealing and extension temp.</td>
<td>61°C</td>
<td>62°C</td>
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</tbody>
</table>
Table 5.1b. The Taqman real-time PCR specifications of the K5054 versus R\textsubscript{low} strain differentiating assay.

<table>
<thead>
<tr>
<th>Assay</th>
<th>K5054 vs. R\textsubscript{low}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG strain</td>
<td></td>
</tr>
<tr>
<td>Gene and GenBank seq. accession #</td>
<td>mge\textsubscript{2}, AY556282</td>
</tr>
<tr>
<td>Forward (F) primer sequence (5’→3’)</td>
<td>CTCAAGAACCAACTCAACCA</td>
</tr>
<tr>
<td>Reverse (R) primer sequence (5’→3’)</td>
<td>GGATTAGGACCAAATTGCGGAT</td>
</tr>
<tr>
<td>Dual labeled probe (P) sequence (5’→3’)</td>
<td>CAACCAGGATTTAATCAACCTCAG</td>
</tr>
<tr>
<td>PCR product size</td>
<td>112bps</td>
</tr>
<tr>
<td>Annealing and extension temp.</td>
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</tr>
</tbody>
</table>
Table 5.1c. The Taqman real-time PCR specifications of the F versus R<sub>low</sub> strain differentiating assay.

<table>
<thead>
<tr>
<th>Assay</th>
<th>F vs. R&lt;sub&gt;low&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
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<td>MG strain</td>
<td></td>
</tr>
<tr>
<td>Gene and GenBank seq. accession #</td>
<td>mge2, AY556230</td>
</tr>
<tr>
<td>Forward (F) primer sequence (5’→3’)</td>
<td>GTTCAAGAACCAAACCTCAACCA</td>
</tr>
<tr>
<td>Reverse (R) primer sequence (5’→3’)</td>
<td>GATTAAGACCGAATTGTTGATTG</td>
</tr>
<tr>
<td>Dual labeled probe (P) sequence (5’→3’)</td>
<td>CAACCAGGATTTAATCAACCTCAG</td>
</tr>
<tr>
<td>PCR product size</td>
<td>112bps</td>
</tr>
<tr>
<td>Annealing and extension temp.</td>
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</table>
Table 5.1d. The Taqman real-time PCR specifications of the st-11 versus R\textsubscript{low} strain differentiating assay.

<table>
<thead>
<tr>
<th>Assay</th>
<th>ts-11 vs. R\textsubscript{low}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG strain</td>
<td></td>
</tr>
<tr>
<td>Gene and GenBank seq. accession #</td>
<td>mge2, AY556232</td>
</tr>
<tr>
<td>Forward (F) primer sequence (5'→3')</td>
<td>CTCAAGAAACCAACTCAACCA</td>
</tr>
<tr>
<td>Reverse (R) primer sequence (5'→3')</td>
<td>GGGGATTAGGAATAAATTGCAGG</td>
</tr>
<tr>
<td>Dual labeled probe (P) sequence (5'→3')</td>
<td>CAGCCAGGATTTAATCAACCTCAG</td>
</tr>
<tr>
<td>PCR product size</td>
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<tr>
<td>Annealing and extension temp.</td>
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</tbody>
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Table 5.1e. The Taqman real-time PCR specifications of the 6/85 versus R\textsubscript{low} strain differentiating assay.

<table>
<thead>
<tr>
<th>Assay</th>
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<th>6/85</th>
<th>R\textsubscript{low}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG strain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene and GenBank seq. accession #</td>
<td>mge2, AY556231</td>
<td>MGA_0319, AY556072</td>
<td></td>
</tr>
<tr>
<td>Forward (F) primer sequence (5’→3’)</td>
<td>CTCAAGAAACCAACTCAACCA</td>
<td>ACTAATAATACTAACCCGCGATAAC</td>
<td></td>
</tr>
<tr>
<td>Reverse (R) primer sequence (5’→3’)</td>
<td>GGATGAGGACCAAATTGCGGAT</td>
<td>GTAGTTGATTCGGTCTACCTGTTT</td>
<td></td>
</tr>
<tr>
<td>Dual labeled probe (P) sequence (5’→3’)</td>
<td>CAGCCAGGATTATACACCTCAG</td>
<td>AAATGGTAACACAGCCAACACCTACTC</td>
<td></td>
</tr>
<tr>
<td>PCR product size</td>
<td>112bps</td>
<td>170bps</td>
<td></td>
</tr>
<tr>
<td>Annealing and extension temp.</td>
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<td>62°C</td>
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Table 5.2. Strain differentiating assay specificity test results, expressed in $C_T$ values.
Specificity was tested by performing each assay’s pair of Taqman real-time PCRs on *in vitro* cultures of the assay’s target strains.

<table>
<thead>
<tr>
<th>Differentiating Assay</th>
<th>MG strain real-time PCR</th>
<th>Reaction’s target strain $C_T$ value</th>
<th>Reaction’s reciprocal strain $C_T$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K5831 vs. $R_{low}$</td>
<td>K5831</td>
<td>12.17</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>$R_{low}$</td>
<td>12.49</td>
<td>Negative</td>
</tr>
<tr>
<td>K5054 vs. $R_{low}$</td>
<td>K5054</td>
<td>12.62</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>$R_{low}$</td>
<td>15.65</td>
<td>Negative</td>
</tr>
<tr>
<td>F vs. $R_{low}$</td>
<td>F</td>
<td>12.01</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>$R_{low}$</td>
<td>12.49</td>
<td>Negative</td>
</tr>
<tr>
<td>ts-11 vs. $R_{low}$</td>
<td>ts-11</td>
<td>15.15</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>$R_{low}$</td>
<td>12.49</td>
<td>Negative</td>
</tr>
<tr>
<td>6/85 vs. $R_{low}$</td>
<td>6/85</td>
<td>14.86</td>
<td>Negative</td>
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<td></td>
<td>$R_{low}$</td>
<td>12.49</td>
<td>Negative</td>
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Table 5.3. Summary of the mean C<sub>T</sub> values, the linear equations and the R-squared values of the study’s Taqman real-time PCRs standard curves.

<table>
<thead>
<tr>
<th>MG strain real-time PCR (target gene)</th>
<th>Mean C&lt;sub&gt;T&lt;/sub&gt; values of template log&lt;sub&gt;10&lt;/sub&gt; copy number</th>
<th>Linear equation</th>
<th>R-squared</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>K5831 &amp; K5054 (mgc2)&lt;sup&gt;∧&lt;/sup&gt;</td>
<td>37.20</td>
<td>33.48</td>
<td>30.48</td>
</tr>
<tr>
<td>F (mgc2)</td>
<td>37.70</td>
<td>34.76</td>
<td>31.19</td>
</tr>
<tr>
<td>6/85 (mgc2)</td>
<td>37.17</td>
<td>33.64</td>
<td>29.97</td>
</tr>
<tr>
<td>ts-11 (mgc2)</td>
<td>36.63</td>
<td>33.41</td>
<td>30.19</td>
</tr>
<tr>
<td>R&lt;sub&gt;low&lt;/sub&gt; (MGA_0319)</td>
<td>38.12</td>
<td>34.80</td>
<td>30.72</td>
</tr>
<tr>
<td>R&lt;sub&gt;low&lt;/sub&gt; (pvpA)</td>
<td>-</td>
<td>33.33</td>
<td>30.44</td>
</tr>
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</table>

<sup>∧</sup> The K5831 and K5054 strains share identical sequence on the amplified region of the mgc2 gene and the same reaction was utilized for both.
Table 5.4. Summary of the MG serology, isolation, and K5831 vs. R_{low} assay results of chickens sampled 33 days post vaccination with the MG K5831 strain A.

<table>
<thead>
<tr>
<th>Vaccine Dose (CCU/ml)</th>
<th>SPA mean grade B</th>
<th>HI log_{10} mean titer</th>
<th>MG isolation C</th>
<th>K5831 strain log_{10} mean copy number D</th>
<th>R_{low} strain log_{10} mean copy number D</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0/12 a</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>6.22 x 10¹</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0/12 a</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>6.22 x 10²</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0/12 a</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>6.22 x 10³</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0/12 a</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>6.22 x 10⁴</td>
<td>0.7 a</td>
<td>0.4 a</td>
<td>4/12 b</td>
<td>0.90 a</td>
<td>0 a</td>
</tr>
<tr>
<td>6.22 x 10⁵</td>
<td>3.3 b</td>
<td>0.2 a</td>
<td>12/12 c</td>
<td>2.83 b</td>
<td>0 a</td>
</tr>
<tr>
<td>6.22 x 10⁶</td>
<td>4.0 b</td>
<td>0.1 a</td>
<td>11/11 c</td>
<td>2.93 b</td>
<td>0 a</td>
</tr>
</tbody>
</table>

A Values within a column with a different lower case superscript are significantly different (P < 0.05)

B Agglutination was scored on a scale 0 to 4; score of 1 considered suspected, and score of 2 or greater was considered positive.

C Number of positive samples/ Number of tested samples

D Copy number values are per reaction; 5 µl of DNA extract solution in a total volume of 25 µl.
Table 5.5. Summary of the MG serology, isolation, air sac lesions, tracheal mucosal width analysis and K5831 vs. R_{low} assay results of chickens sampled 55 days post MG K5831 strain vaccination and 10 days post MG R_{low} strain challenge.

<table>
<thead>
<tr>
<th>K5831 vaccine dose (CCU/ml)</th>
<th>R_{low} challenge</th>
<th>SPA mean grade (^B)</th>
<th>HI log_{10} mean titer</th>
<th>MG isolation (^C)</th>
<th>Air sac lesion score (^C)</th>
<th>Tracheal mucosal mean thickness ((\mu)m)</th>
<th>K5831 strain log_{10} mean copy number (^D)</th>
<th>R_{low} strain log_{10} mean copy number (^D)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>No</td>
<td>0.0 (^a)</td>
<td>1.2 (^a)</td>
<td>0/10 (^a)</td>
<td>0/10 (^a)</td>
<td>70.7 ± 20.5 (^a)</td>
<td>0 (^a)</td>
<td>0 (^a)</td>
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<tr>
<td>None</td>
<td>Yes</td>
<td>2.9 (^{b,c})</td>
<td>2.2 (^b)</td>
<td>10/10 (^b)</td>
<td>10/10 (^b)</td>
<td>173.8 ± 60.6 (^c)</td>
<td>0 (^a)</td>
<td>5.34 (^b)</td>
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<td>3.4 (^c)</td>
<td>2.3 (^b)</td>
<td>10/10 (^b)</td>
<td>10/10 (^b)</td>
<td>252.6 ± 59.8 (^d)</td>
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<td>5.18 (^b)</td>
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<td>2.4 (^b)</td>
<td>10/10 (^b)</td>
<td>10/10 (^b)</td>
<td>151.7 ± 59.2 (^{b,c})</td>
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<td>5.19 (^b)</td>
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<td>9/10 (^b)</td>
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<td>5.12 (^b)</td>
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<td>2.8 (^{b,c})</td>
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<td>10/10 (^b)</td>
<td>186.7 ± 70.6 (^{c,d})</td>
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<td>5/10 (^a)</td>
<td>86.3 ± 13.8 (^{a,b})</td>
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<td>10/10 (^b)</td>
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<td>96.1 ± 19.8 (^{a,b})</td>
<td>1.40 (^{a,b})</td>
<td>1.97 (^c)</td>
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</table>

\(^a\) Values within a column with a different lower case superscript are significantly different \((P \leq 0.05)\)

\(^b\) Agglutination was scored on a scale 0 to 4; score of 1 considered suspected, and score of 2 or greater was considered positive.

\(^c\) Number of positive samples/ Number of tested samples

\(^D\) Results are shown as mean ± SD.
Copy number values are per reaction; 5 µl of DNA extract solution in a total volume of
CHAPTER 6

DISCUSSION

*Human subtlety will never devise an invention more beautiful, more simple or more direct than does Nature, because in her inventions, nothing is lacking and nothing is superfluous.*

(Leonardo da Vinci)

As the smallest and simplest known free-living and self-replicating forms of life it could be argued that, of all Nature’s inventions, the mycoplasmas best fit da Vinci’s description. Alot of scientific attention was directed to the fascinating biology of mycoplasmas and to their role as a minimal life form model. However, the vast majority of the research in the area of mycoplasmology focuses on the human and animal pathogenic mycoplasma species and the diseases that they cause. Avian mycoplasmas were firstly reported over a hundred years ago (3) and still considered as one of the main disease challenges to the poultry industry world wide. Significant progress was made in the area of avian mycoplasmology along the years, but the search for better preventative and control measures pursue. An area of research that laboratories around the world focused on during the last decade was the development of methods to characterize and identify avian mycoplasma (mainly MG) strains and strain variability. Also in this dissertation we focused on the development of MG intraspecific differentiation methods for infection tracking and live vaccine evaluation studies. Another focus of this dissertation was the on going debate whether subclinical mycoplasma infections affect the production and the sensitivity to other disease agents in poultry flocks.
The first study of this dissertation focused on the role of endemic MS infection in US commercial layer complexes, and whether MS challenge at the onset of lay could be associated with the increase in *E. coli* peritonitis mortality reported during the last decade. *E. coli* peritonitis is a common cause of commercial layer mortality and believed to be the number one cause of layer mortality in the US table egg industry during the last decade (Kenton Kreager, personal communication). In order to investigate the possible causes for the increased level of layer *E. coli* peritonitis we had to develop a model that would reproduce the syndrome. The successful reproduction of a typical layer *E. coli* peritonitis disease by the experimental model allowed us to investigate the role that MS might have in this syndrome. A beneficial byproduct of the experimental model was the suggestion of a pathogenesis mechanism for the disease. The direct application of the causative *E. coli* to the upper trachea and the reisolation of an identical *E. coli* strain from the viscera and bone marrow of dead birds with peritonitis are highly suggestive of the respiratory origin of the layer peritonitis syndrome. This observation is in agreement with the industry notion of the respiratory origin of layer peaking period *E. coli* peritonitis (Kenton Kreager, personal communication).

The severity of the tracheal lesions that were induced by the K3344 MS strain brings into question the prevailing assumption of MS as a benign infection in layers. Indeed, the affected chickens appeared clinically normal, but it is likely that birds with such altered tracheas will be more susceptible to respiratory disease agents and environmental contaminants. Our hypothesis is that the injured trachea would allow the avian *E. coli* higher level of colonization and systemic penetration that would result in a higher level of peritonitis.

The experiment demonstrated that the combined challenge of MS plus *E. coli* induced statistically significant mortality relative to the negative controls while the *E. coli* alone did not.
That result implies that the intratracheal *E. coli* challenge by itself is not enough to produce significant peritonitis mortality in layers and that the MS challenge was required to induce the peritonitis mortality. This finding is in agreement with the general notion of colibacillosis as a secondary disease, following a primary infection with respiratory pathogens and/or unfavorable environmental conditions (1). But the above interpretation appears less conclusive when considering the only numerical, and not statistically significant, difference between the mortality in the *E. coli* and the *E. coli* plus MS groups. The difficulty to achieve a more definitive conclusion was the consequent of the relatively low *E. coli* peritonitis mortality in our model, previous models (12), and most clinical cases, and it is questionable whether mortality is the statistically appropriate measurement for this syndrome. The necropsy observation of abundant sub-clinical lesions in the body cavity of MS challenged birds versus no lesions in the solely *E. coli* challenged group is supportive of the significant role of the MS challenge in the layer peritonitis syndrome. It also leads us to think that body cavity sampling (lesion scoring, histology, *E. coli* culture), from 7 days post challenge and onward, could be a better tool for measuring the effect of the different challenges utilized in this experimental model.

This study offers a useful challenge model for the reproduction of layer *E. coli* peritonitis syndrome. The results imply a possible pathogenesis mechanism for the disease which is in agreement with the clinical observations of respiratory origin of the layer *E. coli* peritonitis syndrome. The general notion of colibacillosis as a secondary disease was also demonstrated for the layer *E. coli* peritonitis syndrome, and a virulent MS strain appeared to be a possible primary factor in this syndrome.
The second study of this dissertation describes the first utilization of the single-locus sequence typing (SLST) approach for differentiation of MG isolates. The possibility of using a single variable chromosomal locus with high discriminatory power for differentiation of MG isolates harbors significant advantages over the currently used methods. The freedom from the requirement to culture the organism, the 100% typeability and reproducibility, the quick accumulation of reference database, and the ease and rapid application are the major advantages of a single locus sequencing method. Another important advantage is the ability to perform the SLST analysis as part of the diagnostic MG PCR test. The main disadvantage of the SLST approach is the analysis of a very limited portion of the genome, and when differences are not detected it is required to continue and search for differences with alternative methods before a conclusion can be reached.

The rRNA intergenic spacer region (IGSR) size of most avian mycoplasmas is smaller than five hundred base pairs (bps). Exceptions to this rule are MG and *Mycoplasma imitans*, which contain IGSRs of 660 bps and 2488 bps, respectively (7). Albeit that in several mollicutes very low intraspecific variability of the rRNA IGSR has been reported (11), we hypothesized that the unique MG IGSR is a reasonable place to look for intraspecific genotypic polymorphism. We assumed that a relatively large nonsense genome segment with no reading frame constraints, and with no homologous counterpart sequences in the genome to allow gene conversion repair, could harbor evolutionary accumulation of genotypic changes. We also based our hypothesis on the knowledge that in other prokaryotes intraspecific variations in the lengths and sequences of rRNA IGSR have been described (5, 6, 9, 10).

The results indicated significant sequence variability and good discriminatory power among MG strains by the MG IGSR sequence. The discriminatory ($D$) index of 0.950 was found
to be superior to the previously analyzed MG genes: *gapA*, *MGA_0319*, *mgc2* and *pvpA* with discriminatory indices of 0.713, 0.874, 0.915 and 0.920, respectively (4). Sequence stability of the MG IGSR was demonstrated through numerous *in vitro* passages. The region’s sequence stability, and our accumulating experience in comparing MG IGSR sequences with other genotyping methods (unpublished), led us to conclude that even one base variation is enough indication for isolate differentiation. Another important observation was that none of the study’s strains and field isolates shared the ts-11 vaccine strain MG IGSR sequence. This finding is important in the context that according to our laboratory data several field isolates share the ts-11 *mgc2* sequence (unpublished); the *mgc2* gene PCR is widely used in this laboratory for diagnostic purposes. This superior differentiating capability of the MG IGSR versus the *mgc2* gene could be useful in situations where the involvement of the ts-11 vaccine strain is suspected. This MG IGSR characteristic could address the increased need of the poultry industry for non-culture dependent differentiating tools between vaccine strains and field isolates.

The MG IGSR PCR was highly specific and the amplification of the *M. imitans* rRNA IGSR was expected since these two closely related mycoplasmas having nearly identical rRNA sequences. The size difference between the MG amplicon and the *M. imitans* amplicon is due to a putative transposase insertion in the *M. imitans* 16S-23S rRNA IGSR (7), and makes these two species distinguishable by this reaction.

In summary, the uniquely sized MG 16S-23S rRNA IGSR sequence harbored significant genotypic polymorphism that could be utilized as a preliminary (SLTS) or complementary tool in MG strain differentiation in diagnostic cases and epizootiological studies. The MG IGSR PCR demonstrated high sensitivity and specificity and could be a feasible alternative to the currently available MG PCRs.
The third study of this dissertation focused on the development of a quantitative strain differentiating real-time PCR approach for MG live vaccine evaluation. The evaluation of live avian mycoplasma vaccines and the study of their mechanism of action have lacked the ability to differentially identify and quantify the participating strains within an experimental setup. The available DNA fingerprinting methods (e.g., RAPD, AFLP) are highly dependent on the isolation of the tested organisms in a pure culture (8), and are not suitable for multi strain infection situations. The lack of ability to differentially identify the participating strains imposed significant limitations to the level of control that could be achieved in live vaccine evaluation studies. After the introduction of the vaccine strain, it was no longer possible to accurately verify that only the vaccine strain was present in the experimental birds. In vaccinated bird challenge studies and displacement of field strains by vaccine strain studies, the inability to sensitively detect all the participating strains compromised the soundness of these studies’ conclusions. The mechanism of action of empirically protective vaccine strains could not be well studied without the ability to differentiate and quantify the involved strains in specimens from live birds. The development of the strain differentiating real-time PCR approach addresses the above issues in a highly sensitive, quantitative, and specific manner, as well as, provides investigators with a reliable and a feasible tool for live vaccine evaluation studies.

It is very important to clarify that the differentiation properties of the strain differentiating real-time PCR method is restricted to the known array of strains that were included in the designed assay. The design of this differentiation method was based on sequence differences in unique genomic regions between a known pair of strains. It is most likely that the
targeted sequences that are absolutely different between the assay’s two strains are shared by other MG strains. Such other MG strains will not be differentiated from the assay’s target strain.

The *in vivo* part of the study provided an interesting insight into the live MG vaccine mode of action and protection mechanisms. The post-challenge quantitative evaluation indicated statistically significant lower copy numbers of the challenge strain in the groups that were successfully colonized by the K5831 vaccine and lower copy numbers of both the vaccine and the challenge strains in the group vaccinated with the highest dose of the K5831 vaccine. The lower copy numbers of the challenge strain in the higher dose vaccinated groups correlated well with protection from tracheal and air sac lesions in these groups. These observations could lead to a better understanding of the live MG vaccine protective mechanisms. One hypothesis is that the reduced copy number of the challenge strain is not due to competitive exclusion by the vaccine strain but the outcome of another mechanism. Another hypothesis is that the immune response induced by the non-virulent K5831 strain prevented the *R*<sub>low</sub> challenge strain from massive tracheal colonization and effectively protected against tracheal and air sac lesions. This preliminary data suggests possible protection mechanisms for the K5831 vaccine strain, and demonstrate the plentiful opportunities that the quantitative strain differentiating real-time PCR approach brings to the field of live mycoplasma vaccine research.

This pilot development of a quantitative strain differentiating real-time PCR approach focused on the method’s proof of concept. The real-time PCR with a dual labeled probe technology and the up-to-date real-time PCR equipment allow further improvements of the developed assays. One area for improvement could be the inclusion of the study’s dual reaction assays into a unified multiplex real-time PCR assay, as recently described in closely related
viruses (2). Another possible improvement could be the inclusion of more than 2 strains per assay.

This is the first report of a molecular assay that can absolutely differentiate between a known array of intraspecific mycoplasma strains in a mixed sample. The real-time PCR with a dual labeled probe technology endowed the method with its superior sensitivity, specificity and quantitative properties. The initial application of this quantitative strain differentiating tool was designed for live mycoplasma vaccine evaluation studies and indeed provided a significant upgrade to this area of research. The demonstrated concept of intraspecific differentiating real-time PCR is general and could be considered for a variety of research applications in mycoplasmology and microbiology.

References


