THE EVALUATION OF A LIVE MYCOPLASMA GALLISEPTICUM VACCINE CANDIDATE AND DNA SEQUENCE ANALYSIS IN THE MOLECULAR EPIDEMIOLOGY OF MYCOPLASMA GALLISEPTICUM

by

NAOLA MARSHA FERGUSON

(Under the direction of Stanley H. Kleven)

ABSTRACT

A *Mycoplasma gallisepticum* (MG) isolate from an atypically mild outbreak in turkey breeders was found to be similar to house finch isolates by DNA analyses. A preliminary study in turkeys showed that this isolate (K5054) caused very mild lesions and protected turkeys against subsequent challenge with a virulent MG strain. The safety and efficacy of K5054 was further evaluated in commercial layer-type chickens and turkeys; there was evidence of protection from lesions associated with MG and reduced isolation of R strain post challenge in vaccinated birds. K5054 was further characterized for stability following *in vivo* passages through chickens; the persistence and the duration of immunity elicited by a single vaccination; and the transmissibility to unvaccinated chickens. K5054 has shown promise as a safe, efficacious, stable vaccine with relatively low transmissibility and long persistence and duration of immunity. In another study, MG isolates from the USA, Israel and Australia were characterized by random amplified polymorphic DNA (RAPD) analysis as well as DNA sequence analysis of portions of the phase-variable putative adhesin protein (*pvpA*) gene, the cytadhesin *gapA* gene and an uncharacterized lipoprotein (LP) sequence. The results were compared to reference strains (vaccine and laboratory strains). The RAPD analysis and combined DNA sequence analysis data correlated well, although sequence analysis of any one of the genes did not result in definitive identification of isolates. The Australian isolates appeared to be more similar to the US isolates than were the Israeli isolates.

INDEX WORDS: *Mycoplasma gallisepticum*, K5054, vaccine, poultry, turkey, house finch, atypical, random amplified polymorphic DNA, DNA sequence analysis

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DEDICATION

This dissertation is dedicated to my family, all of whom helped me to accomplish my dreams.

To my father, Theodore, for the opportunity and motivation to pursue my passions fearlessly;

To my mother, Gloria, for being my biggest fan and my biggest inspiration;

To my brothers, Jason and Neron, for helping me to believe that anything is possible;

And to my wonderful husband, Richard, for never ending support and patience.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The Mollicutes (mycoplasmas), the smallest self-replicating organisms, are characterized by their lack of cell wall, small genome size and low G+C content in their genomes. The organisms are highly pleomorphic and naturally resistant to antibiotics affecting cell wall synthesis, for example penicillin (70, 130).

Mycoplasmas are found in multiple hosts, including humans, and many animal species, plants and insects. Mycoplasmas tend to be host specific and have complex nutritional requirements (70). 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 (130).

Mollicutes evolved as a branch of gram-positive bacteria by the process of reductive or degenerative evolution. During this process, the mycoplasmas lost considerable portions of their ancestors' chromosomes but retained the genes essential for life (105, 130).

Unlike other bacteria, all the functions of mycoplasma are expressed from relatively limited gene sets. Mycoplasmal genomes have so far revealed few of the complex systems for classic gene regulation and environmental sensing found in other bacteria. Mycoplasmas do however have systems that provide variation in the expression and structure of specific gene products. These systems include localized mutable

sequences in specific genes or gene sets and the ability to revert to alternative phenotypes through reversible mutations. The result is that adaptation of variants within the population is a primary strategy for survival (130).

Tremendous weight has been given to 16S rDNA sequences in the phylogeny, taxonomy and species identification of Mollicutes (17, 48, 49, 93, 124-127, 142). The Mollicutes have been divided into 5 phylogenetic units by 16S rRNA sequencing (143), including the acholeplasmas, the anaeroplasmas, the spiroplasmas, the mycoplasmas and ureaplasmas.

Numerous avian mycoplasmas have been described but those recognized as pathogens of domestic poultry include *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae, Mycoplasma meleagridis,* and *Mycoplasma iowae*. The most economically significant of these pathogens is MG, which causes respiratory disease in chickens, turkeys and other avian species (69).

The disease, in chickens known as chronic respiratory disease (CRD) and turkeys as infectious sinusitis, can result in respiratory rales, coughing and nasal discharge, as well as sinusitis in turkeys. Airsacculitis may cause significant economic losses at processing, there may also be egg production losses, reduced feed efficiency and medication costs (85).

Mild or subclinical cases of MG, termed ‘atypical’ infections, have been observed in chickens and turkeys (22, 47, 102, 152). MG infections in turkeys resulting in mild clinical disease are unusual. 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 (85). These atypical infections are often difficult to diagnose (8, 59).

Although MG infection occurs naturally in chickens and turkeys, the organism has also been isolated from naturally occurring infections in other avian species (85). The significance of these species in the epidemiology of MG has not been established although wild passerine species may act as biological carriers (73, 119). This may be a relatively minor factor in the overall epidemiology of MG.

In early 1994, an epidemic of MG began in wild house finches (*Carpodacus mexicanus*) in the mid-Atlantic United States (88). MG had not been previously associated with clinical disease in wild passerine birds. The disease has become widespread and has been reported throughout the eastern United States and Canada (30). Molecular characterization of isolates 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 (87).

MG can be transmitted horizontally by direct or indirect contact. 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 (20). Carrier birds, including backyard flocks, are thought to be the main source of MG outbreaks (27). MG can also be transmitted vertically *in ovo*. The highest frequency of transmission occurs during the acute phase of the disease, but transmission may also occur at a lower rate during chronic infection (43, 44).

Pathogenesis and Virulence Factors

The clinical manifestations of severe MG infection in chickens and turkeys is generally due to a complicated etiology involving concurrent infections and environmental factors (68, 85). Colibacillosis, live vaccines, and immunosuppression may all affect the severity of the disease (46, 113, 116).

Most mycoplasmas adhere tightly to the epithelial linings of the respiratory and urogenital tract, rarely invading tissues, and are considered surface parasites. Adhesion is 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 (79, 129).

Early in MG infection in the upper respiratory tract there is release of mucous granules, destruction and exfoliation of ciliated and nonciliated epithelial cells (23). Ciliostasis has been observed *in vitro* (2).

Pathogenic effects that may be attributable to mycoplasma infection include damage to host cell membranes, clastogenic and oncogenic effects. Adhesion to host cells, membrane fusion, cell invasion, stimulation or suppression of the host immune response and antigenic variation may be important factors in mycoplasma disease pathogenesis.

Mildly toxic by-products of mycoplasma metabolism, such as superoxide and hydrogen peroxide, may be involved in oxidative damage to host cells membranes in mycoplasma infections (130).

It has also been theorized that mycoplasma-associated preferential loss of potassium channels occurs resulting in depolarization of the cell membrane leading to the ciliostasis observed in mycoplasma infected ciliary cells (52).

MG shares similar pathogenic mechanisms with two human mycoplasmas, *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. These mycoplasmas share a flask-shaped morphology characterized by a unipolar terminal organelle that is involved in mucosal attachment and gliding motility.

The molecular basis of mycoplasma pathogenicity remains largely elusive. The clinical picture of mycoplasma infections in humans and animals is suggestive of damage due to host immune and inflammatory responses rather than direct toxic effects by mycoplasmal cell components. Various mycoplasmal virulence factors have been described but there appears to be no clear causal relationship between these factors and pathogenicity (130).

Antigenic variation and phenotypic switching. Antigenic variation or phenotypic switching refers to the ability of a microbial species to alter the antigenic character of its surface components. These surface organelles are the major targets of the host antibody response; therefore the ability of a microorganism to rapidly change the surface antigenic repertoire and consequently to vary the immunogenicity of these structures allows effective avoidance of immune recognition. The molecular switching events leading to the generation of phenotypic variants are generally reversible.

During their evolution and adaptation to a parasitic mode of life, the mycoplasmas have developed various genetic systems providing a highly plastic set of variable surface proteins. The majority of surface proteins involved in generating antigenic variation in

mycoplasmas are lipoproteins. The generation of a versatile surface coat through high-frequency phase and size variation provides the organism with a useful tool for immune system avoidance, allowing the mycoplasma to escape antibody attack (130).

Many pathogenic mycoplasmas are able to undergo surface variation resulting in an antigenic shift (11, 41, 53, 118, 133, 134). Epitope switching has been observed for many MG surface molecules (13, 32, 84).

The high degree of phenotypic variation exhibited by mycoplasmas is considered a major factor in pathogenicity and chronic infection of the host (128, 132, 135, 155). Changes in surface topology of MG during host infection and molecular characteristics of several MG surface proteins have been described (13, 32).

Adhesion and cytadhesins. The cytadhesion process of mycoplasmas appears to be multifactorial, involving a number of accessory membrane proteins. These act in concert with cytoskeletal elements to facilitate the lateral movement and concentration of the adhesion molecules at the attachment tip organelle. Extensive analysis of the cytadherence process in *M. pneumoniae* has demonstrated that this involves the coordinate action of primary adhesin molecules (P1 and P30) in concert with an array of high-molecular-weight accessory membrane proteins (76).

MG cytadhesins that have been identified include LP64 (31), pMGA (109), PvpA (14, 154), MGC1 (45, 56), MGC2 (51), and MGC3 (123, 156).

Antigenic variation of cytadhesins allow MG to escape the host immune system (6).

A surface lipoprotein known as pMGA (106) is expressed in abundance by MG. The pMGA family of hemagluttinins (adhesins) probably plays an important role in

colonization and chronicity of respiratory disease in the avian host. Although each MG usually expresses only one homogenous, unique pMGA molecule (40), this lipoprotein appears to exhibit high frequency phase and antigenic variation during culture (especially when growing in media containing anti-pMGA antibodies) (107) and during the course of a natural infection (39).

A switch to off expression of pMGA occurs during the acute stages of infection, a second switch to antigenic variants occurs during the chronic stage of infection. The variation in expression results from switches in expression of different members of a repertoire of genes (9, 108, 109).

The number of pMGA gene copies present in the genome varies from 32 to 70 in different strains (9). Despite the presence of multiple copies of the gene, only one individual gene is expressed at a time in a given strain. All but one of the genes is transcriptionally silent. The control of transcription of each member of the gene family resides in a short GAA trinucleotide repeat region that lies 18 bases 5' to the -35 box of the promoter of each gene (38, 98).

PvpA is postulated to be one of the accessory membrane proteins of MG. Variation within PvpA could affect the specificity or affinity of adherence. It is an integral membrane surface protein with a free C terminus that is subject to spontaneous high-frequency phase variation in expression and exhibits size variation among strains (14, 154). It exists as a single chromosomal copy. PvpA variation of expression is controlled at the level of translation. A localized nonsense mutation in a poly-GAA tract of the *pvpA* coding region was shown to determine PvpA antigenic variation.

Another type of variation shown to occur with PvpA results from deletions within the 3' end of the *pvpA* gene and causes size variation of PvpA polypeptide. 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 and within two direct repeat sequences (14),. Tthis domain may be under selective pressure in the host. Several MG strains differing in their adherence and pathogenicity have varying deletions and sizes of PvpA. Analysis of *pvpA* has been used to differentiate between MG strains (99).

The *mgc1* gene (56), also referred to as *gapA* (45), is one of three clustered genes with adhesin-related functions. It encodes a protein with homology to the P1 cytadhesin protein of *M. pnuemoniae*. Immunoblot analysis of various strains has demonstrated intraspecies variation in the size of GapA (98, 105 and 110 kDa) (45).

The other two genes in the cluster are *mgc2* (51) and *crmA* (123) (also referred to as *mgc3* (156)). MGC2 is a 32 kDa protein with homology to P30 of *M. pnuemoniae*, the *mgc2* gene is located upstream of the *gapA* gene. CrmA (or MGC3) is a 120kDa cytadherence associated membrane protein, the gene is located downstream of the *mgc1* gene (156).

CrmA is cotranscribed with GapA, they interact and are essential for cytadherence (121). CrmA is encoded by the second gene in the g*apA* operon and shares significant sequence homology to the ORF6 gene of *M.pneumoniae*, which has been shown to play an accessory role in the cytadherence process. GapA and CrmA have been shown to undergo concomitant phase variation; the underlying genetic mechanism is a reversible base substitution resulting in a nonsense mutation in the *gapA* gene that affects the expression of *gapA* and the *crmA* gene located downstream (149).

Intracellular location. Human and animal mycoplasmas were shown to be taken up by polymorphonuclear leukocytes (110). More recently MG was shown to have the ability to invade human epithelial cells and chicken embryo fibroblasts *in vitro.* This has been proposed as a mechanism of resisting host defenses and antibiotic therapy, as well as the method that MG uses to establish chronic infections and cause systemic infections (10, 112, 150).

The invasion of mycoplasma into the host cell cytoplasm may affect cell function and integrity. Lysis of human lung fibroblasts (111) and cell disruption and necrosis (100) have been demonstrated with *M. genitalium* and *M. penetrans*, respectively.

Immune system modulation. Mycoplasmas stimulate both a specific and a non-specific immune response in the host. The specific anti-mycoplasma reactions have been shown to play a role in the development of lesions and the exacerbation of disease. The non-specific responses induced by mycoplasmas include the suppression or polyclonal stimulation of B and T lymphocytes, induction of cytokines, increasing the cytotoxicity of macrophages, natural killer cells and T cells, and activating the complement cascade. The ability of mycoplasmas to modulate the host response may allow them to evade or suppress host defenses (130).

Immune response

Birds that recover from MG induced disease have some degree of immunity but remain carriers of the organism (12). The immunogenicity of MG strains varies and is correlated with virulence (79, 94).

Birds lacking a fully functional immune system (neonatal thymectomy or bursectomy) have significantly higher lesion scores than normal birds following MG infection (140). It seems that both antibody and cell mediated immunity are important in the host response to MG (19).

Although the bursa and bursal derived cells have been shown to be essential for a protective immune response to MG (3, 77), it has also been shown that there is a poor correlation between systemic antibody levels and protection from challenge (97, 117).

Chickens produce a protective immune response to MG that seems to be localized to the respiratory tract. MG antibodies in upper and lower respiratory tract washes have been shown to prevent attachment and establishment of MG in tracheal organ cultures (7) and *in vivo* (140, 151).

A significant leukocyte migration into the mucosa of the upper respiratory tract is a hallmark of MG infection. The 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 (151) and serum, as well and leukocyte migration into the mucosa (19).

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 (140).

Although a lymphproliferative response in the respiratory tract is a prominent feature of disease induced by MG, the cell mediated immune response has not been extensively investigated. It has recently been shown that there is specific stimulation of $CD8^+$ cells, particularly in the acute phase of the disease (35). It is postulated that the

fusion of mycoplasma membranes with the host cells enables presentation to $CD8^+$ lymphocytes.

Diagnosis

Serological screening is routinely used as an indicator of MG infection. Sera commonly are analyzed for MG antibodies using the serum plate agglutination (SPA) test, a hemagglutination-inhibition (HI) test and an enzyme-linked immunosorbent assay (ELISA) test (69). The SPA test is rapid, sensitive and inexpensive but may result in non-specific reactions (5, 15, 42), so that reactors must generally be confirmed by the HI or ELISA tests. Serum dilution has also been used to reduce non-specific reactions (136). The HI test is less sensitive but more specific than the SPA test. It is however, a time consuming 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 (58, 60).

MG infection is generally confirmed by the isolation and identification of MG or by DNA based detection methods (83, 92). 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 (69).

Mycoplasma isolates are commonly identified using direct and indirect immunofluorescence (69, 138). Mycoplasma species-specific hyperimmune sera is an essential reagent for these tests and may limit the ability of some laboratories to perform the test (69).

MG species-specific PCR (78, 114), PCR-RFLP (34, 65) and oligonucleotide probe (29, 33) techniques have been developed.

During the acute stage of the infection the number of organisms in the upper respiratory tract is high (80, 151); however in chronic infection the number of organisms is much lower and routine methods may not detect MG (83).

In some situations it may be very difficult to isolate MG consistently from infected flocks. These instances include chronic MG cases and infections with strains of low pathogenicity (69, 152). The overgrowth of non-pathogenic mycoplasmas may also interfere with cultivation of MG from clinical samples in the laboratory (104).

The isolation rates of fastidious MG strains may be enhanced *in vivo* by bioassays (103). 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.

Control of MG

Control 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 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. Inactivated MG vaccines have been widely used in several countries. The results with MG bacterins have been variable (50, 54, 55, 63); they protect against loss of egg production in layers (25, 43, 44), but do not prevent infection or provide consistent protection against respiratory disease (1).

Live MG vaccines. One of the options for control is live MG vaccines (67, 83, 145). 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 (72, 82).

Live vaccines that are currently used worldwide to control MG include F strain (Schering Plough, Kenilworth, N.J.) (4, 101), 6/85 (Intervet America, Millsboro, Del.) (24) and ts-11 (Bioproperties, Inc., Australia, marketed in the US by Merial Select Laboratories, Gainsville, GA.) (146, 147).

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 (145).

It has been established that there is a complex relationship between infectivity, pathogenicity and immunogenicity of MG strains (79). It has been established that virulence, invasiveness and immunogenicity of MG strains are directly correlated (94). Some live vaccines may be so mild 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, 96).

F strain vaccine has been used extensively (92), it is very immunogenic and mildly virulent in chickens (1, 16, 131), but too virulent for use in turkeys (95, 96). F strain has been associated with MG outbreaks in commercial turkeys (86). It is effective in displacing virulent (field) strains from poultry operations (72, 74, 82).

6/85 and ts-11 vaccines have both been shown to elicit protective immunity in chickens and to possess little or no virulence for chickens and turkeys (24, 146, 147).

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, 91). The duration of immunity elicited by a live vaccine may be associated with the colonization and persistence of the vaccine in the respiratory tract.

Although 6/85 has been reported not to persist in the trachea and to be poorly transmissible, “6/85-like” isolates have been recovered from vaccinated and unvaccinated contact chickens long after vaccination (137) as well as from unvaccinated turkeys (Kleven, unpublished).

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 (145). Experimental passage of 6/85 through chickens and turkeys did not result in a substantial increase in virulence (24, 26). Attempts to serially passage ts-11 in birds were unsuccessful but the vaccine appeared to retain its characteristics after three passages (147).

F strain may be more virulent than 6/85 or ts-11, but it provides better protection against airsacculitis and persists at higher levels in the upper respiratory tract (1). F strain also protects against colonization by more virulent challenge strains (21) and is capable of displacing endemic field strains (72, 74). However, the persistence and transmissibility of F strain means that it can be isolated from farms long after vaccination has ceased. There is the potential for spread to susceptible poultry (66), most significantly, turkeys (86).

F strain is readily transmissible to unvaccinated pen mates and chickens in adjacent pens (66) and can be isolated from farms long after vaccination has ceased and has been implicated outbreaks in commercial turkeys (86). 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 (37).

The ts-11 and 6/85 vaccines have both been shown to be poorly transmissible to in contact poultry (90, 147).

The distinct advantage of the milder vaccine strains over F strain is their lack of virulence in turkeys and their low transmissibility (24, 91, 95, 147). The ts-11 vaccine

may be useful in displacing endemic F strain in poultry complexes as part of an eradication program (141).

Although 6/85 has been reported to transmit poorly (91), not to persist in the respiratory tract for long periods (72), and to be apathogenic (24), there have been reports of MG outbreaks in unvaccinated turkeys and chickens from which "6/85-like" MG strains have been isolated (137).

The vaccination of turkeys against MG has not been shown to be feasible although there has been limited use of 6/85 (83).

A GapA-negative high passage MG R strain (GT 5) has recently been described as a potential modified live vaccine (122, 123).

The characteristics of different live MG vaccines have been described and compared extensively (1, 91, 145). The choice of vaccine should be carefully evaluated in each situation.

Epidemiology and Strain Differentiation

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 (120).

Methods of strain differentiation must have high differentiation 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 (135).

Intraspecies heterogeneity and antigenic variability can be observed in mycoplasmas through serological testing (75, 139) and electrophoresis of cell proteins (62).

The shortcomings of phenotypically based typing methods, such as those based on a reaction with an antibody (135), 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 MG strains restriction fragment length polymorphisms (RFLP) of DNA (64, 71), DNA and ribosomal RNA gene probes (61, 153), and PCR with strain-specific primers (115).

Random amplified polymorphic DNA (RAPD). The most widely used method for differentiating MG strains is random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR, analysis (18, 28, 36). The RAPD assay was first described by Williams et al. (148) and Welsh and McClelland (144). 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 epidemiological studies (57, 81, 87, 89).

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 MG subtype may be present. Furthermore, technical factors such as target DNA/primer ratio may significantly impact the reproducibility of RAPD patterns.

DNA sequence analysis. 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 with 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 (14, 154), MGC1 (45, 56), MGC2 (51), and MGC3 (123, 156), have been described. The DNA sequences of these genes are under great selective pressure and may be useful in the molecular epidemiology of MG.

Objectives of the Research

With increasing concentrations of poultry in restricted geographic areas and large multiple age complexes, the control of MG by purchasing MG-free stock and keeping flocks MG-free has become much more difficult. There is an increasing need to use live vaccines to control MG in these situations. Live vaccines prevent production losses by allowing controlled exposure of flocks to avirulent MG strains resulting in the development of immunity to subsequent field challenges. Live vaccines can also be part of an eradication program by displacing the resident virulent MG strain (72, 141); cessation of vaccination should allow the flocks to return to an MG-free status.

The properties of an ideal MG vaccine include avirulence, immunogenicity, life-long protection, affordability, easy methods of administration and stability. Unfortunately, although each of the currently available vaccines has its advantages, none of them attains the ideal status in every respect (145). F strain is highly efficacious but moderately virulent in chickens and unsafe for turkeys. 6/85 and ts-11 are both avirulent and immunogenic, but the level and duration of protection elicited by these milder

vaccines may not be as good as F strain, 6/85 more so than ts-11. Also, none of the currently available vaccines are used in turkeys. Turkeys are very susceptible to MG infection and more severely affected than chickens. F-strain is too virulent for use in turkeys. Although there has been restricted use of 6/85, MG isolates similar to this strain ("6/85-like") have been isolated from clinically ill commercial turkeys (Kleven, unpublished). The ts-11 appears to be incapable of infecting turkeys (Kleven, personal communication).

There is therefore a need for an avirulent, immunogenic, and stable MG vaccine that is safe for chickens and turkeys.

With the widespread use of live vaccines there is also an increasing need to differentiate between vaccine strains and field isolates. Methods to easily and clearly differentiate between pathogens and vaccine strains are necessary to avoid confusion. Epidemiology and strain differentiation is an essential part of eradication and control of MG. Precise information about the origin of infectious agents allows cost-effective control programs to be targeted to the weak points of the current system. This approach should be more economical than shotgun approaches of control programs. Molecular epidemiology should aid in pinpointing the source of outbreaks, identify biological carriers, and increase understanding about the transmission and maintenance of MG in the environment.

RAPD analysis is a widely used, rapid, sensitive and effective tool in the molecular epidemiology of MG but this method has its drawbacks and difficulties. Chief among them is the need for pure cultures of the target organism, as well as difficulty in the reproducibility and standardization between laboratories.

Alternative methods of molecular epidemiology should allow the construction of a database that allows easy comparison of an unknown isolate to all the known strains in the database. RAPD analysis restricts the number of strains to which an unknown is compared. The low reproducibility of RAPD analysis makes the comparison of patterns from different RAPD reactions or different agarose gels unreliable.

An alternative method should also allow MG strain discrimination at the level of clinical samples so avoiding the MG isolation step that is necessary for RAPD analysis.

Nucleotide sequence analysis of a specified gene (s) may allow the development of a PCR that is performed directly on clinical samples to detect MG and identify strains. The building of a sequence database allows the comparison of many seemingly unrelated isolates. This method would be reproducible and easily standardized. We must determine the discriminatory power of this method with respect to selected genes that have shown intraspecies variability and may be useful in this technique. To be useful DNA sequencing should be able to differentiate between MG strains.

The aim of the studies described in Chapters 2, 3 and 4 was to evaluate a naturally occurring MG isolate as a live vaccine in poultry. The aim of the study described in Chapter 5 is to evaluate DNA sequence analysis of selected genes as a method of molecular epidemiology of MG.

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CHAPTER 2

CHARACTERIZATION OF A NATURALLY OCCURRING INFECTION OF A MYCOPLASMA GALLISEPTICUM HOUSE FINCH-LIKE STRAIN IN TURKEY BREEDERS[1]

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Key words: *Mycoplasma gallisepticum*, turkey, house finch, atypical

Abbreviations: ELISA = enzyme-linked immunosorbent assay; HI = hemagluttination inhibition; MG = *Mycoplasma gallisepticum*; PCR = polymerase chain reaction; RAPD = random amplified polymorphic DNA; SPA = serum plate agglutination

Summary.

An outbreak of *Mycoplasma gallisepticum* (MG) in commercial turkeys involving very mild clinical signs was difficult to confirm by routine methods. In the first part of this study, (Trial A), a bioassay was conducted to increase the likelihood of detecting MG. Susceptible turkeys were inoculated with sinus exudates from four different affected commercial turkey flocks. They were evaluated for clinical signs, as well as by serology and culture of tracheal swabs at 21 and 42 days post challenge. An MG isolate from one of the sinus exudates used for inoculation, designated K5054, was very similar to isolates from house finches when characterized by random amplified polymorphic DNA (RAPD) analysis as well as DNA sequence analysis of portions of the phase-variable putative adhesin protein (*pvpA*) gene, a lipoprotein gene (LP), and the cytadhesin *gapA*/*mgc1* gene. The turkeys inoculated with the K5054 sinus exudate seroconverted in the absence severe clinical signs. There was a single re-isolation of K5054 from these turkeys 42 days post challenge. Susceptible contact turkeys were co-mmingled with the K5054-inoculated turkeys at 49 days post challenge. There was no evidence of transmission of MG to the contacts by culture or serology at 7, 21 or 35 days after co-mingling. In the second part of this study, (Trial B), the contacts and K5054 sinus exudate -inoculated turkeys from Trial A were challenged with virulent R strain 88 days after the K5054 sinus exudate inoculation. On necropsy 10 days post challenge, the evaluation of gross and microscopic lesions, serology and culture showed that the turkeys previously inoculated with K5054 sinus exudate were protected against disease and re-infection.

Introduction

Mycoplasma gallisepticum (MG) infection may result in chronic respiratory disease of chickens and infectious sinusitis of turkeys. Although the incidence of MG infection has decreased significantly in the past years due to extensive control programs within the poultry industry, MG remains an important concern (26).

Control of MG has been based on the eradication of the organism from breeder flocks and biosecurity to maintain the mycoplasma-free status in breeders and their progeny. Serological monitoring is performed periodically and isolation of MG or DNA based detection methods (e.g. polymerase chain reaction (PCR) (22)) are used to confirm the presence of MG (23, 26).

In some situations it may be very difficult to isolate MG consistently from infected flocks. These instances include chronic MG cases or infections with strains of low pathogenicity (19, 38). The overgrowth of non-pathogenic mycoplasmas may also interfere with cultivation of MG from clinical samples in the laboratory (32).

The isolation rates of fastidious MG strains may be enhanced *in vivo* by bioassays (31). 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.

MG infections in turkeys resulting in mild clinical disease are unusual. Turkeys are often more severely affected by MG infections than chickens; turkeys may develop severe sinusitis, airsacculitis, and tendovaginitis (26). Mild or subclinical cases of MG, termed ‘atypical’ infections, have been observed naturally and experimentally in chickens

and turkeys (7, 13, 30, 38). These atypical infections are often difficult to diagnose (2, 18).

Although MG infection occurs naturally in chickens and turkeys, the organism has also been isolated from naturally occurring infections in other avian species (26). The significance of these species in the epidemiology of MG has not been established although it has been suggested that wild passerine species may act as biological carriers (20, 34).

In early 1994, an epidemic of MG began in wild house finches (*Carpodacus mexicanus*) in the mid-Atlantic United States (25). MG had not been previously associated with clinical disease in wild passerine birds. The disease has become widespread and has been reported throughout the eastern United States and Canada (9). Molecular characterization of isolates suggested that the house finch epidemic arose from a single source and that the MG infection has not been shared between songbirds and commercial poultry (24).

In this case report, a bioassay was conducted in susceptible turkeys in an effort to increase the likelihood of detecting MG from commercial turkeys experiencing an atypical outbreak with very mild clinical signs (Trial A). MG isolates made during the bioassay were characterized by molecular methods. In a subsequent trial, (Trial B), some of the turkeys from Trial A were challenged with a virulent MG strain.

Case History

The MG outbreak occurred in September 2000 on a turkey breeder farm in northern Indiana. The farm consisted of four houses containing approximately 5000 birds

each. The hen flock involved was 39 weeks old. The turkeys were routinely monitored for MG according to the NPIP serological surveillance program, which consisted of serum plate agglutination (SPA) screening with hemagglutination inhibition (HI) confirmation of plate reactors. Prior to and at the onset of clinical signs, no serological positives were detected on the farm with this protocol.

The MG infection was detected when the breeder farm manager noticed a slight increase in mortality in one house. The mortality problem was diagnosed as fowl cholera, but during that visit a very small number of birds were observed to have swollen infraorbital sinuses. No respiratory noise was heard in the flock. Tracheal swabs taken at this time were PCR positive for MG but mycoplasma was not isolated from these first samples. The SPA and HI tests were still negative at this time. All four houses were depopulated within two weeks of the diagnosis. Although the serum plate test did show some reactors two weeks after the onset of clinical signs, most of these reactors did not have geometric mean titers higher than 1:20 on the HI test, although there were a few titers of 1:40.

Sera from July, August, and September were then tested with an ELISA test (IDEXX, Westbrook, Maine). From those results it was surmised that the infection had started in early to mid-August. Commercial flocks with poults from the hen flock involved that were placed on or after September 11th, 2000 were eventually positive in an age dependent fashion (the youngest poults seroconverted first and the oldest poults seroconverted last). The breeder farm may have had more infected hens laying infected eggs when the last flocks were sourced. Unfortunately, we did not have an isolate from

the poults to confirm vertical transmission from the breeders although this was more than likely.

Another interesting observation in the commercial flocks was that most sisters to the positive toms never seroconverted. They were more than likely to be infected since the toms were positive, but they may not have had time to seroconvert. Most hens were processed at 13 weeks and toms at 19 weeks. One heavy hen flock (19wks) did become serologically positive.

The MG infection in the commercial turkeys did not adversely affect the processing of the affected flocks. The turkeys were prophylactically treated with antibiotics. The producer was able to return to normal operations within a year, despite having farms with multiple ages of turkeys on each farm.

Materials and Methods

Turkeys. Forty turkeys (Hybrid, Ontario, Canada) were acquired at one day of age from a commercial source. The turkeys were housed in groups of eight on the floor in 3X3 m^2 colony houses with pine shaving litter.

Serology. Sera were analyzed for MG antibodies using the serum plate agglutination (SPA) test, a hemagglutination-inhibition (HI) test and an enzyme-linked immunosorbent assay (ELISA) test.

The SPA and HI test were carried out according to procedures described by Kleven (19). The SPA test was conducted using commercial antigen (Intervet America, Millsboro, Del). The agglutination was scored from 0 (negative) to 4 (strong, rapid reaction). An agglutination score $\geq$ 1 was considered positive. Antigen prepared from the

A5969 MG strain and turkey erythrocytes were used in the HI test. A titer of 1:20 was considered suspect and $\geq$ 1:40 positive.

The ELISA test was performed using a commercial test kit for turkeys (IDEXX, Westbrook, Maine).

Isolation and identification of mycoplasma. Cotton swabs from trachea and air sacs, as well as sinus exudates were used for culture. They were inoculated in Frey's modified broth and agar and incubated at 37^0C. Mycoplasma isolates were identified using direct immunofluorescence (19).

PCR. MG PCR was performed on the sinus exudates used for challenge as well as at 42 days post challenge on sinus exudate and pooled samples of tracheal swabs. The PCR was carried out using primers and a procedure described by Lauerman (22) that was modified so that the PCR reaction mixture consisted of 32µl of water ultra-pure, 5µl of 10X PCR buffer, 1µl dNTP (10mM), 1µl F Primer (50 µM), 1µl R Primer (50 µM), 1µl Taq DNA polymerase (5 U/µl), 4µl magnesium chloride (25mM) and 5µl sample (100-2000ng DNA).

Random amplified polymorphic DNA (RAPD) analysis. MG isolates were analyzed using random amplified polymorphic DNA (RAPD) analysis. The procedure and primers used were described by Fan *et al.* (8). RAPDs were also conducted using primers described by Geary *et al.* (10) and Charlton *et al.* (5).

DNA sequence analysis. DNA base sequences of the *pvpA* gene (GenBank accession number AF224059) (3) of the isolates and reference strains were compared as described by Liu *et al.* (27) using a polymerase chain reaction with primers 3R and 4F (given as *pvpA* 1F and *pvpA* 2R). DNA sequences from a PCR product from a lipoprotein

gene (LP) described by Nascimento *et al.* (33), GenBank accession number AF075588, using primers LP-1F (GGA TCC CAT CTC GAC CAC GAG AAA A) and LP-2R (CTT TCA ATC AGT GAG TAA CTG ATG A) were also compared. Sequences corresponding to positions 30-541 were analyzed. Finally, sequences from the *mgc1* gene (17), GenBank accession number U34842, using primers Adhs1-3F (TTC TAG CGC TTT ARC CCT AAA CCC) and Adhs1-4R (CTT GTG GAA CAG CAA CGT ATT CGC) were compared. Positions 3823-4154 were compared. This gene is analogous to a gene known as *gapA* (12). The amplified products of these PCR's were sequenced at the Molecular Genetics Instrumentation Facility (MGIF), University of Georgia. Sequence analysis was performed with MegAlign (DNASTAR, Lasergene, Inc. Madison, Wisconsin).

Evaluation of lesions. The lesions in turkeys necropsied during the study were evaluated grossly by air sac lesions scoring on a scale from 0 to 4 (21). The tracheal lesions were evaluated microscopically by measuring the width of the tracheal mucosa. A section was collected from the upper third of the trachea (approximately 1 inch distal to the larynx) and fixed in 10% neutral formalin. The tracheal mucosa thickness was measured at four equidistant points on histological slides of cross sections of tracheas (37).

Experimental design. Trial A. Four groups of 8 turkeys were challenged with sinus exudates collected from affected commercial turkeys on four different farms. A fifth group of 8 served as negative controls. The sinus exudates used to challenge the turkeys were cultured for mycoplasma and PCR analysis was performed. The turkeys were observed for clinical signs, bled for serology, and tracheal swabs were obtained for

culture at 21 and 42 days post challenge. Sinus exudate from one bird with mild sinusitis was also cultured at 42 days post challenge.

At 49 days post challenge, one group of turkeys (previously inoculated with sinus exudate from which K5054 was isolated) was commingled with 8 susceptible turkeys (the negative control group). The remaining 24 turkeys in the groups inoculated with sinus exudates from other farms were sacrificed at this time. The remaining turkeys were observed for clinical signs, bled for serology, and tracheal swabs were obtained for mycoplasma culture at 7, 21 and 35 days after commingling (i.e. 56, 70 and 84 days post inoculation with K5054). Two of the contact turkeys were necropsied 56 days post challenge (7 days after co-mingling). Swabs of the tracheas and air sacs were obtained for culture. The remaining turkeys were used in Trial B.

Trial B. Eighty-eight days after the sinus exudate inoculation, 7 of the turkeys (4 principals and 3 contacts) were challenged via fine aerosol (21) with the previously characterized R strain of MG (35). The 7 remaining turkeys (4 principals and 3 contacts) were used as controls. All of the turkeys were necropsied 10 days after the R strain challenge. They were bled for serology, tracheal and air sac swabs were obtained for culture, and air sac lesions were scored. Tracheal sections were fixed in formalin for tracheal mucosa thickness measurements.

Turkeys in this study were euthanized with carbon dioxide according to the animal care and use policies of The University of Georgia.

Statistical analysis. Air sac lesion scores were analyzed using the Kruskal-Wallis Rank Sums test. The mean tracheal mucosa thickness was analyzed using the Tukey-

Kramer HSD test. (JMP® Statistics Made Visual, SAS Institute Inc., SAS Campus Drive, Cary, NC 27513).

Results

Trial A. MG was isolated from one of the sinus exudates used to challenge one of the groups of turkeys in Trial A; the isolate was designated K5054.

The turkeys inoculated with sinus exudates from the 3 other farms did not show any evidence of MG infection throughout the study. They were negative by serology, culture and PCR throughout the study. No MG was detected by culture or PCR in the sinus exudates used to inoculate these turkeys.

The serological responses of the turkeys in the group challenged with K5054 sinus exudate and the negative controls are shown in Table 2.1. The turkeys challenged with the K5054 sinus exudate developed MG antibody titers by 21 days post challenge. The antibody titers peaked at 56 days post challenge (7 days after co-mingling with contacts) and declined over time. The negative controls and contact turkeys were serologically negative by HI and ELISA throughout the study. At 70 and 84 days post challenge (21 and 35 days after co-mingling), 1 and 2 of the contact turkeys were weakly positive (agglutination score = 1) by the SPA test.

A single turkey in the group challenged with K5054 sinus exudate had unilaterally swollen infraorbital sinus at 42 days post challenge. The sinusitis resolved by the end of the trial. There were no other clinically affected turkeys observed. The 2 contact turkeys necropsied 56 days post challenge (7 days after co-mingling) showed no gross lesions associated with MG infection.

MG was recovered a single time from the turkeys during this trial at 42 days post challenge. The isolate (K5074B) was made from sinus exudate collected from the turkey with unilateral sinusitis. No mycoplasma was isolated from tracheal swabs obtained at 21, 42, 56, 70 and 84 days post inoculation.

The sinus exudates used to challenge the susceptible turkeys were negative for MG by PCR for all the groups, including the sinus exudate from which K5054 was eventually cultured. PCR analysis of the K5054 culture was positive. Tracheal swab samples obtained at 42 days post challenge were negative by PCR.

The RAPD gel patterns of the sinus exudate isolate (K5054), and the isolate made during the trial (K5074B), were similar to each other and to that of the house finch isolates when RAPD analyses were performed (Fig. 2.1).

DNA sequencing of the *pvpA*, LP and *gapA*/*mgc1* genes showed that K5054 and K5074B had sequence identities of 100% when compared to the house finch isolates of MG for all three genes.

Trial B. The results of this trial are summarized in Tables 2.2 and 2.3. All of the challenged turkeys responded serologically. The highest titers were in the contact group that was challenged with R strain. The principals (K5054 inoculated) that were challenged with R strain had higher titers than the principals that were not challenged. The contacts that were not challenged remained serologically negative.

MG was isolated from the tracheas and air sacs of all the turkeys in the group of contacts that were challenged with R strain. RAPD analysis confirmed that the recovered isolates were R strain. No MG was recovered from the other groups.

The mean air sac lesion score of the challenged contact group was significantly higher than those of all of the other groups. The mean tracheal mucosa thickness was also significantly higher for this group ($P \leq 0.05$). None of the other groups showed a significant difference in mean air sac lesion score or mean tracheal mucosa thickness ($P \leq 0.05$).

Discussion

MG isolates from poultry that exhibit reduced pathogenicity, transmissibility and immunogenicity have been described as atypical or variant. Most of these reports have been in chickens, but there are also reports from turkeys (7, 13, 30, 38). Atypical serology, very mild or absent clinical signs and lesions, as well as difficulty in making an isolation by routine culture methods have been reported. In these instances the confirmation of suspicious cases is very difficult.

In this instance, routine monitoring for MG according to the NPIP serological surveillance program did not detect the MG infection in the hen flock until late in the outbreak, although the ELISA test showed MG titers in banked sera.

The K5054 isolate in this study was characterized as similar to the house finch isolates by multiple analyses. Close examination of RAPD patterns using primer sets described by Fan *et al.* (8) and Geary *et al.* (10), revealed that the patterns of K5054 and house finch isolates are not completely identical but very similar. Previous studies have established that the finch MG strain was clearly different from commercial poultry strains, vaccines and lab strains (24).

Wild house finches nest and feed around buildings and farms, so that they may be in contact with domestic poultry. The house finch strain has been shown not to be host-specific to the finch although transmission may be slow. There is growing evidence that songbird species other than house finches are susceptible to MG infection and disease (14, 15, 29). MG from house finches can also be transmitted from affected finches to chickens by natural (contact) methods but transmit to domestic poultry quite slowly and with little pathogenic effect (34, 36).

Inoculation of turkeys with K5054 sinus exudate in this study resulted in seroconversion with the appearance of very mild clinical signs 42 days after the challenge. This suggests that K5054 is mildly pathogenic in turkeys, although the inoculum dose in the bioassay may have been very low. PCR was unable to detect MG in the sinus exudates used to inoculate the turkeys.

The K5054 strain appeared to be relatively fastidious as are the house finch isolates (28). This may be a characteristic of the K5054 strain in combination with minimal replication of the MG in the experimental turkeys.

The infection did not spread among the turkeys in the bioassay. This suggests that this strain was not as highly transmissible between turkeys as with MG in house finches. It has been suggested that the MG of finches naturally has a low rate of transmission and that the rate of transmission is density-dependent (16) resulting in the seasonal fluctuation of the house finch MG outbreak (6).

The K5054 infection in the turkeys challenged in the study may never have reached the titers necessary for transmission to the contacts. There were some weakly positive SPA tests in the contact birds, which may have been due to non-specific

reactions (1, 4, 11). The SPA positives disappeared in later samplings and were never confirmed by the HI or ELISA tests.

It has been shown that minimal biosecurity measures that restrict contact between domestic poultry and house finches should significantly reduce the potential for MG transmission between these species (34, 36).

The K5054 strain may be different from the MG isolates from house finches in its ability to spread to and infect domestic turkeys. There may be future isolations of MG house finch-like strains from commercial poultry as the characteristics of house finch strain changes over time. On the other hand, this infection may have occurred under extreme or unusual circumstances, making it an incidental finding. However, there were no obvious confounding circumstances (e.g. moribund finches) observed at the farm.

From the trials it appears that infection with the K5054 strain results in positive serology with very mild clinical disease; confirmation of the infection by isolation of MG may be difficult. The infection appears to spread very slowly between turkeys. This was mirrored in the clinical picture of K5054 in the commercial turkeys.

There was a significant difference in resistance to subsequent MG infection and disease when the K5054 sinus exudate-inoculated turkeys were challenged with virulent R strain. These results indicate that although the K5054 strain appears to be mildly virulent in turkeys it is immunogenic enough to provide protection against disease.

This may be the first recorded incident of a naturally occurring infection of a house finch-like MG strain in commercial poultry.

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Table 2.1.Trial A. Serological responses of turkeys at 21, 42, 56, 70 and 84 days post challenge with K5054 sinus exudate. Principals (K5054 challenged) and contacts were co-mingled at 49 days post challenge.

Group	Days post challenge	SPA	HI	ELISA
K5054	21	7/8[A] (1.6)[B]	6/8 [A] (33.6)[C]	ND
Negative Controls	21	0/8 (0.0)	0/8 (0.0)	ND
K5054	42	8/8 (2.3)	4/8 (28.2)	7/8 [A] (1.0±0.6)[D]
Negative Controls	42	0/8 (0.0)	0/8 (0.0)	0/8 (0.1 ± 0.0)
K5054	56	8/8 (2.9)	8/8 (61.7)	8/8 (2.0 ± 1.0)
Contacts	56	0/8 (0.0)	0/8 (0.0)	0/8 (0.6 ± 0.2)
K5054	70	7/7 (1.3)	0/8 (0.0)	8/8 (1.0 ± 0.6)
Contacts	70	2/6 (0.3)	0/6 (0.0)	0/6 (0.0 ± 0.1)
K5054	84	7/8 (1.5)	4/8 (9.2)	4/8 (0.7 ± 0.9)
Contacts	84	1/6 (0.2)	0/6 (0.0)	0/6 (0.0 ± 0.0)

[A] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, and ELISA: ≥ 0.6)

[B] Mean agglutination grade (0 to 4).

[C] Geometric mean titer.

[D] Geometric mean S/P ratio ± standard deviation

ND = not done

Table 2.2. Trial B. Serological responses of principal (K5054 challenged) and contact turkeys 10 days post challenge with R strain.

Group	Challenge	SPA	HI	ELISA
Principals	Yes	4/4[A] (2.3)[B]	4/4 [A] (56.6)[C]	4/4 [A] (2.2 ± 1.4)[D]
	No	ND	3/4 (33.6)	1/4 (0.6 ± 0.3)
Contacts	Yes	3/3 (2.7)	3/3 (160.0)	3/3 (2.3 ± 0.3)
	No	ND	0/3 (0.0)	0/3 (0.0 ± 0.0)

[A] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, ELISA: ≥ 0.6)

[B] Mean agglutination grade (scored from 0 to 4).

[C] Geometric mean titer.

[D] Geometric mean S/P ratio ± standard deviation

Table 2.3. Trial B. Air sac and tracheal lesion evaluation and rate of isolation of *M. gallisepticum* from the trachea and air sacs of principal (K5054 challenged) and contact turkeys 10 days post challenge with R strain.

Group	Challenge	Air sac lesion score [A]	Mean tracheal mucosa thickness[B]	MG isolation	
				Trachea	Air sacs
Principals	Yes	0/0 [C] (0.0± 0.0)[Da]	14.44 ± 2.68[a]	0/4 [C a]	0/4 [C a]
	No	0/0 (0.0 ± 0.0)[a]	13.25 ± 1.97[a]	0/4[a]	0/4[a]
Contacts	Yes	3/3 (4.0 ± 0.0)[b]	31.25 ± 1.39[b]	3/3[b]	3/3[b]
	No	0/0 (0.0 ± 0.0)[a]	14.33 ± 0.63[a]	0/3[a]	0/3[a]

[A] Grossly scored from 0 to 4

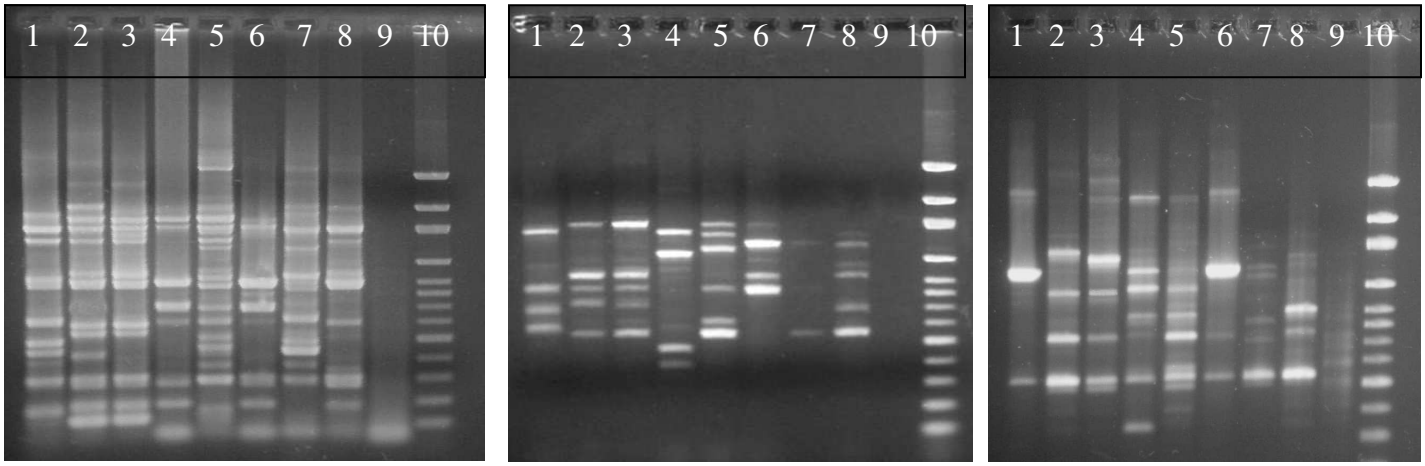
[B] Mean thickness for the group in units. 1 unit = 0.0083mm

[C] No. of positive samples/No. of tested samples (Air sac score ≥ 1)

[D] Mean score ± standard deviation

[a,b] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

Fig. 2.1. RAPD gel patterns of MG isolates using primers described by a) Fan *et al.*, b) Geary *et al.*, and c) Charlton *et al.* Lane 2 = HF-51 (house finch isolate); Lane 3 = K5054. Lanes 1,4,5,6, 7, 8 = Reference strains R, ts-11, 6/85, F, S6 and A5969 respectively. Lane 9= Negative control; lane 10 = 100bp molecular weight marker.


1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

CHAPTER 3

SAFETY AND EFFICACY OF THE AVIRULENT MYCOPLASMA GALLISEPTICUM STRAIN K5054 AS A LIVE VACCINE IN POULTRY[2]

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[2] N.M. Ferguson, V.A. Leiting and S.H. Kleven. Submitted to *Avian Diseases*, 04/19/2003

Key words: *Mycoplasma gallisepticum*, K5054, vaccine

Abbreviations: ELISA = enzyme-linked immunosorbent assay; MG = Mycoplasma gallisepticum; HI = hemagglutination inhibition; RAPD = random amplified polymorphic DNA; SPA = serum plate agglutination

Summary

A *Mycoplasma gallisepticum* (MG) isolate from an atypically mild outbreak in turkey breeders was found to be similar to house finch isolates by DNA analyses. A preliminary study in turkeys showed that this isolate (K5054) caused very mild lesions and protected turkeys against subsequent challenge with a virulent MG strain. In this study, K5054 was further evaluated as a potential vaccine strain in commercial layer-type chickens (Trial 1) and turkeys (Trial 2). The safety of K5054 was evaluated by aerosol challenge followed by evaluation of gross and histopathological lesions, as well as serological reactions and isolation of MG from the trachea and air sac. Infection of chickens and turkeys with K5054 resulted in little evidence of MG lesions. There was weak seroconversion and K5054 was consistently re-isolated from the tracheas of chickens and turkeys. The efficacy of K5054 as a vaccine was evaluated by aerosol challenge of vaccinated chickens and turkeys with virulent R strain. There was evidence of protection from lesions associated with MG and reduced isolation of R strain post challenge in vaccinated birds.

Introduction

Mycoplasma gallisepticum (MG) is a significant pathogen causing respiratory disease in chickens and turkeys worldwide (28). In most countries control of MG is based on maintaining breeding stock free of infection, but MG vaccines are used in areas where this is not feasible (25). Large poultry populations in small geographic areas and multiple-age farms that never depopulate make eradication and control of MG by biosecurity alone difficult. In these situations inactivated and live vaccines have also been used to control MG.

Eradication of MG is preferable to vaccination wherever possible; 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 (20, 24).

Inactivated MG vaccines have been widely used in several countries. The results with MG bacterins have been variable (13, 14, 15, 17); they do protect against loss of egg production in layers (7, 10, 11), but do not prevent infection or provide consistent protection against respiratory disease (1).

Live vaccines that have been used to control MG include F strain (2, 32), and more recently, 6/85 (6) and ts-11 (38, 39).

It has been established that there is a complex relationship between infectivity, pathogenicity and immunogenicity of MG strains (23). Studies have indicated that the level of protection elicited by live vaccines is directly correlated with the virulence of the vaccine strain (1, 31).

F strain is very immunogenic but mildly virulent in chickens (1, 4, 34). It has been shown to be effective in displacing virulent MG strains from poultry operations (20, 24).

F strain is too virulent for use in turkeys (30, 31), and has been associated with MG outbreaks in commercial turkeys (26).

6/85 and ts-11 vaccines have both been shown to elicit protective immunity in chickens and to possess little or no virulence for chickens or turkeys (6, 38, 39). They are both poorly transmitted to unvaccinated birds, and do not persist long in the upper respiratory tract (1, 27).

Vaccination of turkeys against MG has not been shown to be feasible, although there has been restricted use of 6/85 (25).

In this study chickens and turkeys were vaccinated/challenged with an avirulent MG isolate from commercial turkeys (K5054) to investigate the safety of this strain. The vaccinated chickens and turkeys were subsequently challenged with a virulent MG strain to evaluate the efficacy of K5054 as a live vaccine.

Materials and Methods

Chickens and turkeys. Two hundred and thirty-five layer-type unsexed chickens (Hy-line, West Des Moines, Iowa) were obtained from a commercial hatchery at one day of age. They were divided into 4 groups and housed in Horsfal isolator units under positive pressure.

One hundred and four female turkeys (Hybrid, Ontario, Canada) were acquired at one day of age from a commercial source. The turkeys were housed together in floor pens (1.5x3 m^2) until 3 weeks of age when they were divided into 3 treatment groups and moved to different 3x3 m^2 colony houses with pine shaving litter.

MG strains and isolates. The K5054 isolate was obtained from sinus exudate of a commercial turkey flock (9). This flock did not exhibit the typical clinical signs of MG infection. This isolate was found to be similar to house finch strains by random amplified polymorphic DNA (RAPD) analysis and DNA sequence analysis of the phase-variable putative adhesin protein gene (*pvpA*) (3), a lipoprotein gene (LP) (33), and the *gapA/mgc1* gene (12, 16).

The ts-11 vaccine (Merial Select, Gainesville, GA) is commercially available and was used according to the manufacturer's directions.

R strain is a virulent MG strain the characteristics of which have already been described (34).

Serology. Sera were analyzed for MG antibodies using the serum plate agglutination (SPA) test using commercial antigen (Intervet America, Millsboro, Del). The hemagglutination-inhibition (HI) test was performed using antigen prepared from A5969 strain and chicken erythrocytes.

The SPA and HI test were carried out according to procedures described by Kleven (19). Commercial enzyme-linked immunosorbent assays (ELISA) were also performed on the sera (IDEXX, Westbrook, Maine).

An SPA score $\geq$ 1 was considered positive. An HI titer of 1:20 was considered suspect and $\geq$ 1:40 was considered positive. A geometric mean sample/positive (S/P) ratio of $\geq$ 0.6 on the ELISA test was considered positive.

Isolation and identification of mycoplasma. Cotton swabs from trachea and air sacs were used for culture. They were inoculated in Frey's modified broth and agar and

incubated at 37^0C. Mycoplasma isolates were identified using direct immunofluorescence (19).

Random amplified polymorphic DNA (RAPD) analysis. MG isolates were analyzed using RAPD analysis. The procedure and primers used were described by Fan et al. (8). A minimum of 20% of all of the MG isolates recovered after vaccination or challenge were typed by this method.

Evaluation of lesions. Gross lesions were evaluated by air sac lesion scoring on a scale from 0 to 4 (22). The tracheal lesions were evaluated microscopically by measuring the width of the tracheal mucosa. A section of the upper third of the trachea (approximately 1 inch distal from the larynx) was fixed in 10% neutral formalin. The tracheal mucosa thickness was measured at four equidistant points on histological slides of cross sections of tracheas (37).

Experimental design. Two separate trials were conducted in this study. Trial 1 was conducted using chickens and Trial 2 using turkeys. Each trial had a safety phase and an efficacy phase.

Trial 1. Two hundred and thirty-five commercial layer-type chickens were divided into 4 groups at 3 weeks of age. One group of 66 chickens was vaccinated/challenged with ts-11 vaccine by eye drop (1.1×10^4 color changing units (CCU)/ml). Another group of 66 was vaccinated/challenged with K5054 (1.6×10^8 CCU/ml). A third group of 15 chickens was challenged with the virulent MG R strain (1.9×10^8 CCU/ml) (positive controls). The K5054 and R challenges were administered by coarse spray (22) using a commercial paint sprayer (Preval® Sprayer Division, Precision Valve Corporation, Yonkers, NY). Approximately 1ml of actively growing

culture was sprayed per bird. A fourth group of 48 unvaccinated, unchallenged chickens served as negative controls.

In the safety phase of this trial, 16 chickens from each of the ts-11, K5054 and negative control groups were removed, necropsied and evaluated along with the 15 positive controls (challenged with R strain) at 10 days post vaccination/challenge, They were bled for serology, the trachea and air sacs were cultured, the air sac lesions were scored and a section of trachea removed for histological examination.

In the efficacy phase of Trial 1, at five weeks post challenge/vaccination the chickens remaining from the first part of the trial (including 40 challenge controls that were not previously exposed to MG) were challenged with R strain by coarse spray. The negative controls, 10 chickens from the K5054 group, and 10 chickens from the ts-11 group were not challenged and kept as unchallenged controls. All of the birds were necropsied and examined 10 days post challenge as described above.

Trial 2. One hundred and four turkeys were divided into 3 groups at 4 weeks of age. One group of 35 was vaccinated/challenged with K5054 (1.6×10^8 CCU/ml) and 15 turkeys from the challenge control group were challenged with R strain (1.9×10^8 CCU/ml); both were administered by coarse spray. The third group of turkeys served as negative controls. In the safety phase of this trial, 15 turkeys from each of the vaccinated/challenged groups and 14 negative controls were necropsied at 10 days post challenge. They were evaluated by serology, culture of trachea and air sacs, air sac lesion scoring and measurement of the tracheal mucosa. In the efficacy phase of Trial 2, at 6.5 weeks post vaccination/challenge, the remaining turkeys were challenged with R strain

(with the exception of the negative controls). These turkeys were necropsied at 10 days post challenge and evaluated as before.

The experimental designs of Trials 1 and 2 are summarized in Tables 3.1 and 3.2, respectively.

Chickens and turkeys that died or were euthanized for humane reasons during the study were examined for gross lesions of MG.

Chickens and turkeys in this study were euthanized by cervical dislocation or with carbon dioxide according to the animal care and use policies of The University of Georgia, Athens, GA.

Statistical analysis. Air sac lesion scores were analyzed using the Kruskal-Wallis Rank Sums test. The mean tracheal mucosa thickness was analyzed using the Tukey-Kramer HSD test. JMP® Statistics Made Visual (SAS Institute Inc., SAS Campus Drive, Cary, NC 27513).

Results

The chickens and turkeys in Trials 1 and 2 did not seroconvert strongly, with the exception of the birds that were challenged with R strain. The serological data for the trials are summarized in Tables 3.3, 3.4, 3.5 and 3.6.

Four chickens died during Trial 1, three that were vaccinated with ts-11 and one that was vaccinated with K5054. There were no gross lesions of mycoplasmosis associated with these deaths.

Two turkeys were euthanized during Trial 2 due to broken wings. Both of these turkeys had been vaccinated with K5054. There were no gross lesions of MG in either of the turkeys.

The results of the safety components of Trials 1 and 2 are summarized in Tables 3.7 and 3.8, respectively. The mean lesion scores of chickens in the ts-11 and K5054 groups in Trial 1 were not significantly different from the negative controls ($P \leq 0.05$). Similarly, the mean lesion scores of the turkeys in Trial 2 that were vaccinated/challenged with K5054 were not significantly different from the negative controls ($P \leq 0.05$). The isolates that were typed by RAPD analysis were identified as ts-11, K5054 and R strain corresponding to the respective vaccination/challenge. K5054 was re-isolated from 100% of the tracheal cultures in Trials 1 and 2, but from only 6 of 16 (37.5%) of the air sac cultures of chickens in Trial 1, and 13 of 15 (86.7%) of the air sac cultures of turkeys in Trial 2.

The results of the efficacy components of Trials 1 and 2 are summarized in Tables 3.9 and 3.10, respectively. The mean lesion scores of the K5054 vaccinated chickens and turkeys after challenge were not significantly different from the negative controls in both trials ($P \leq 0.05$). MG was isolated from significantly fewer K5054-vaccinated chickens that were challenged as compared to unvaccinated chickens and turkeys that were challenged ($P \leq 0.05$). This was also true for tracheal samples from K5054-vaccinated turkeys as compared to unvaccinated turkeys that were challenged in Trial 2 ($P \leq 0.05$). The isolates recovered from groups that were challenged with R strain were identified as R strain by RAPD analysis. K5054 was re-isolated 6 weeks post vaccination from 10 of

10 (100%) of the tracheal swabs from K5054-vaccinated chickens that were not challenged.

Discussion

The properties of an ideal MG vaccine include avirulence, immunogenicity, life-long protection, affordability, easy methods of administration and stability. Unfortunately, although each of the currently available vaccines has advantages, none of them attains the ideal status in every respect (37).

The characteristics of different live MG vaccines have been described and compared extensively (1, 27, 37). The choice of vaccine should be carefully evaluated in each situation.

F strain may be more virulent than 6/85 and ts-11, but it provides better protection against airsacculitis and persists at higher levels in the upper respiratory tract (1). F strain also protects against colonization by more virulent challenge strains (5) and is capable of displacing endemic field strains (20, 21). However, the persistence and transmissibility of F strain means that it can be isolated from farms long after vaccination has ceased. There is the potential for spread to susceptible poultry (18), most significantly, turkeys (26).

The distinct advantage of the milder vaccine strains over F strain is their lack of virulence in turkeys and their low transmissibility (6, 27, 30, 39). The ts-11 vaccine may be useful in displacing endemic F strain in poultry complexes as part of an eradication program (36).

Although 6/85 has been reported to transmit poorly (27), not to persist in the respiratory tract for long periods (20), and to be apathogenic (6), there have been reports

of MG outbreaks in unvaccinated turkeys and chickens from which “6/85-like” MG strains have been isolated (35).

It has been established that virulence, invasiveness and immunogenicity of MG strains are directly correlated (29). Some live vaccines may be so mild 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.

In this study, infection with K5054 and ts-11 did not result in significant clinical signs or lesions indicative of MG disease. It can be concluded that these strains are safe vaccines.

In the group of chickens vaccinated with ts-11, MG was isolated infrequently from the trachea, and not at all from the air sacs. Strain K5054 was consistently re-isolated from the tracheas and less consistently from the air sacs of chickens and turkeys vaccinated with K5054. This may be indicative of the colonization and persistence of K5054 in the upper respiratory tract of chickens and turkeys.

R strain was re-isolated less frequently from birds that were vaccinated prior to challenge than those that were unvaccinated, although the difference was only significant with respect to the turkeys in Trial 2 ($P \leq 0.05$). Vaccination may prevent subsequent infection with a virulent MG strain.

The evidence gathered in this preliminary study indicates that the K5054 strain is relatively avirulent and immunogenic in chickens and turkeys. Further study is warranted to determine the characteristics of K5054 with respect to those of a good live MG vaccine candidate, but this study is evidence of significant potential.

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Table 3.1. Trial 1 - experimental design.

		Safety Phase		Efficacy Phase	
Treatment Group	No. of chickens	Vaccine/ Challenge [A]	No. of chickens	Challenged (R strain)[B]	No. of chickens
ts-11	66	ts-11	16	Yes	40
				No	10
K5054	66	K5054	16	Yes	40
				No	10
Challenged controls	55	R	15	Yes	40
Negative controls	48	None	16	No	32

[A] Chickens were vaccinated/challenged at 3 weeks of age by eye drop (ts-11) or coarse spray (K5054 and R strain); they were examined 10 days post vaccination/challenge.

[B] Chickens were challenged with R strain by coarse spray at 8 weeks of age (i.e., 5 weeks post vaccination); they were examined 10 days post challenge.

Table 3.2. Trial 2 - experimental design.

		Safety Phase		Efficacy Phase	
Treatment Group	No. of turkeys	Vaccine/ Challenge[A]	No. of turkeys	Challenged (R strain)[B]	No. of turkeys
K5054	35	K5054	15	Yes	20
Challenged controls	35	R	15	Yes	20
Negative controls	34	None	14	No	20

[A] Turkeys were vaccinated/challenged at 4 weeks of age by coarse spray with K5054 or R strain; they were examined 10 days post vaccination/challenge.

[B] Turkeys were challenged with R strain by coarse spray at 10.5 weeks of age (i.e., 6.5 weeks post vaccination); they were examined 10 days post challenge.

Table 3.3.Trial 1 (Safety). Serological responses of chickens after challenge with ts-11, K5054 or the R strain at 3 weeks of age [A].

Challenge strain	Days post challenge	SPA	HI	ELISA
ts-11	10	2/16[Ba] (0.1)[C]	0/16[a] (0.0)[D]	0/16[a] (0.8 ± 0.0)[E]
K5054	10	2/16[a] (0.1)	0/16 [a] (0.0)	1/16 [a] (0.3 ± 5.1)
R	10	15/15 [b] (3.0)	5/15 [b] (20.6)	11/15 [b] (0.9 ± 0.7)
Neg. Controls	10	0/16[a] (0.0)	0/16 [a] (0.0)	0/16 [a] (0.4 ± 0.0)
ts-11	35	8/10 [a] (2.4)	1/10 [a] (11.8)	7/10 [a] (0.9 ± 0.9)
K5054	35	10/10 [a] (3.0)	3/10 [a] (24.6)	3/10 [a,b] (0.3 ± 0.2)
R	35	N/A	N/A	N/A
Neg. Controls	35	0/5[b] (0.0)	0/5 [a] (0.0)	0/5[b] (0.2 ± 0.0)

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, and ELISA: ≥ 0.6)

[C] Mean agglutination grade (from 0 to 4).

[D] Geometric mean titer

[E] Geometric mean S/P ratio ± standard deviation

N/A - not applicable

Table 3.4. Trial 2 (Safety). Serological response of turkeys 10 days after challenge with K5054 or the R strain at 4 weeks of age [A].

Challenge strain	SPA	HI	ELISA
K5054	0/15[Ba] (0.0)[C]	0/15[a] (0.0)[D]	0/15[a](0.44 ± 0.01)[E]
R	15/15[b] (3.1)	0/15[a] (2.2)	3/15[b] (0.24 ± 0.49)
Neg. Controls	0/14[a] (0.0)	0/14[a] (0.0)	0/14[a] (0.68 ± 0.05)

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, and ELISA: ≥ 0.6)

[C] Mean agglutination grade (0 to 4).

[D] Geometric mean titer

[E] Geometric mean S/P ratio ± standard deviation

Table 3.5. Trial 1 (Efficacy). Serological response of chickens 10 days after challenge with the R strain of MG at 8 weeks of age [A].

Group	Challenged	SPA	HI	ELISA
ts-11	Yes	38/38[Ba] (4.0)[C]	37/38[a] (43.8)[D]	38/38[a](4.6 ± 1.1)[E]
	No	9/9[a] (3.8)	0/9[b] (20.0)	8/9[a] (1.3 ± 0.8)
K5054	Yes	39/39[a] (4.0)	32/39[c] (37.3)	39/39[a] (2.9 ± 1.1)
	No	10/10[a] (4.0)	1/10[b] (15.9)	10/10[a] (1.3 ± 0.4)
No Vaccine	Yes	40/40[a] (4.0)	33/38[a,c] (36.5)	40/40[a] (3.4 ± 1.0)
	No	0/32[a] (0.0)	0/32[b] (0.0)	0/32[b] (0.1 ± 0.1)

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, and ELISA: ≥ 0.6)

[C] Mean agglutination grade (0 to 4)

[D] Geometric mean titer.

[E] Geometric mean S/P ratio ± standard deviation

Table 3.6. Trial 2 (Efficacy). Serological response of turkeys 10 days after challenge with the R strain at 6 weeks post vaccination [A].

Group	Challenged	SPA	HI	ELISA
K5054	Yes	18/18[Ba](1.3)[C]	1/18[a] (2.4)[D]	17/18[a](1.6±0.7)[E]
No Vaccine	Yes	20/20[a] (2.0)	18/20[b] (47.0)	20/20[a] (1.3 ± 0.4)
No Vaccine	No	0/20[b] (0.0)	0/20[a] (0.0)	0/20[b] (0.1 ± 0.1)

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, and ELISA: ≥ 0.6)

[C] Mean agglutination grade (0 to 4)

[D] Geometric mean titer.

[E] Geometric mean S/P ratio ± standard deviation

Table 3.7. Trial 1 (Safety). Lesion scores and MG isolation from chickens 10 days after challenge with ts-11, K5054 or the R strain at 3 weeks of age [A].

Challenge strain	Air sac lesions [B]		Tracheal mucosal thickness[C]	MG isolation[D]	
				Trachea	Air sacs
ts-11	0/16[E]	$(0.0 \pm 0.0)^{Fa}$	6.38 ± 1.77^{a}	1/16 [Ea]	0/16 [Ea]
K5054	0/16	$(0.0 \pm 0.0)^{a}$	5.94 ± 1.77^{a}	$16/16^{b}$	$6/16^{b}$
R	12/15	$(1.8 \pm 1.15)^{b}$	42.75 ± 1.83^{b}	$15/15^{b}$	$15/15^{c}$
Neg. Controls	0/16	$(0.0 \pm 0.0)^{a}$	6.23 ± 1.77^{a}	$0/16^{a}$	$0/16^{a}$

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] Macroscopically scored from 0 to 4

[C] Mean thickness for the group in units. 1 unit = 0.0083mm

[D] MG isolates recovered from groups challenged with R strain, ts-11 or K5054 were identified as R strain, ts-11 or K5054 respectively by RAPD analysis.

[E] No. of positive samples/No. of tested samples (Air sac score $\geq$ 1)

[F] Mean score $\pm$ SD

Table 3.8. Trial 2 (Safety). Air sac and tracheal lesion evaluation and MG isolation from turkeys 10 days post challenge with K5054 or the R strain at 4 weeks of age [A].

Challenge strain	Air sac lesions [B]	Tracheal mucosal thickness[C]	MG isolation[D]	
			Trachea	Air sacs
K5054	2/15 [E] (0.2 ± 0.7)[Fa]	12.77 ± 2.52[a]	15/15 [Ea]	13/15 [Ea]
R	13/15 (2.2 ± 1.3)[b]	20.87 ± 4.36[b]	15/15[a]	15/15[a]
Neg. Controls	0/14 (0.0 ± 0.0)[a]	8.29 ± 1.02[a]	0/14[b]	0/14[b]

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] Macroscopically scored from 0 to 4

[C] Mean thickness for the group in units. 1 unit = 0.0083mm

[D] MG isolates recovered from groups challenged with R strain or K5054 were identified as R strain or K5054 respectively by RAPD analysis.

[E] No. of positive samples/No. of tested samples (Air sac score ≥ 1)

[F] Mean score ± SD

Table 3.9. Trial 1 (Efficacy). Mean lesion scores and MG isolation from chickens 10 days after challenge with the R strain of MG at 6 weeks of age [A].

Group	Challenged	Air sac lesions[B]	Tracheal mucosal thickness[C]	MG isolation[D]	
				Trachea	Air sacs
ts-11	Yes	17/38 [E] (0.7 ± 1.0)[Fa]	16.32 ± 19.27[a]	38/38 [a]	33/38 [a]
	No	0/9 (0.0 ± 0.0)[b]	7.28 ± 1.77[a]	5/9[b]	0/9 [b]
K5054	Yes	13/39 (0.4 ± 0.5)[a]	8.88 ± 2.91[a]	39/39[a]	36/39[a]
	No	0/10 (0.0 ± 0.0) [b]	7.50 ± 1.72[a]	10/10[a]	0/10 [b]
No Vaccine	Yes	40/40 (2.9± 0.4)[c]	57.23 ± 22.17[b]	40/40[a]	40/40[c]
	No	0/32 (0.0 ± 0.0)[b]	6.94 ± 1.42[a]	0/32[c]	0/31[b]

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] Macroscopically scored from 0 to 4

[B] Mean thickness for the group in units ± standard deviation. 1 unit = 0.0083mm

[C] MG isolates from groups challenged with R strain were all identified as R strain by RAPD analysis; MG isolates from groups vaccinated with ts-11 or K5054 and not challenged with R strain were identified as ts-11 or K5054 respectively by RAPD analysis.

[D] No. of positive samples/No. of tested samples (Air sac score ≥ 1)

[E]Mean score ± SD

[a,b.c] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

Table 3.10. Trial 2 (Efficacy). Mean lesion scores and MG isolation from turkeys 10 days after challenge with the R strain at 10.5 weeks of age [A].

Group	Challenged	Air sac lesions[B]	Tracheal mucosal thickness[C]	MG isolation[D]	
				Trachea	Air sacs
K5054	Yes	12/18[E] (1.4 ± 1.3)[Fa]	13.90 ± 2.10[a]	14/18[Ea]	11/18[Ea]
Challenged Controls	Yes	20/20 (3.8 ± 0.4)[b]	22.06 ± 3.46[b]	20/20[b]	20/20[b]
Negative Controls	No	0/20 (0.0 ± 0.0)[a]	13.55 ± 2.00[a]	0/20[c]	0/20[c]

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$

[B] Macroscopically scored from 0 to 4

[C] Mean thickness for the group in units. 1 unit = 0.0083mm

[D] MG isolates from groups challenged with R strain were all identified as R strain by RAPD analysis.

[E] No. of positive samples/No. of tested samples (Air sac score ≥ 1)

[F] Mean score ± SD

CHAPTER 4

FURTHER STUDIES OF AN AVIRULENT MYCOPLASMA GALLISEPTICUM STRAIN AS A LIVE VACCINE IN POULTRY[3]

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Key words: *Mycoplasma gallisepticum*, K5054, vaccine

Abbreviations: ELISA = enzyme-linked immunosorbent assay; HI = hemagglutination inhibition; MG = Mycoplasma gallisepticum; RAPD = random amplified polymorphic DNA; SPA = serum plate agglutination

Summary

A *Mycoplasma gallisepticum* (MG) isolate (K5054) showed promise as an efficacious and safe live vaccine in poultry in previous studies. In this study, K5054 was further characterized in three trials. In Trial 1, the stability of K5054 was evaluated by *in vivo* passages through chickens. An isolate from the tenth chicken passage (K5383-2) was used to challenge chickens. The virulence of the chicken-passaged isolate was not significantly increased when compared to the parental K5054 isolate. In Trial 2, the persistence and the duration of immunity elicited by a single vaccination with K5054 were evaluated. Chickens vaccinated with K5054 at 3 weeks of age were evaluated over a seven-month period with 5 periodic samplings for serology and tracheal culture for mycoplasma. Ten of the vaccinated chickens were removed at each sampling period and challenged with virulent R strain. K5054 persisted in the trachea and the vaccinated chickens were protected from disease for the duration of the experiment. In Trial 3, the transmissibility of K5054 to unvaccinated chickens was evaluated. Seeders were inoculated with K5054 via eye-drop and placed so that there were groups of chickens in direct contact (within the same pen) and indirect contact (adjacent or with an empty pen separating). R strain was used as a control in a similar experimental design. Tracheal culture and serological monitoring of chickens from each group of contacts evaluated the transmissibility of K5054 and R strain. K5054 did not transmit to in contact chickens.

Introduction

Mycoplasma gallisepticum (MG) is a significant pathogen that affects poultry worldwide. The control of MG is generally by isolation and maintenance of breeding stock free of MG. Live MG vaccines have been used to control MG in areas where isolation of poultry flocks and eradication is not feasible (15, 21, 31). Eradication of MG is preferable to vaccination wherever possible, and 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 (17, 20, 30).

Live vaccines that are currently used worldwide to control MG include F strain (Schering Plough, Kenilworth, N.J.) (26), 6/85 (Intervet America, Millsboro, Del.) (5) and ts-11 (Bioproperties, Inc., Australia, marketed in the US by Merial Select Laboratories, Gainsville, GA.) (32).

An MG isolate, designated K5054, from an atypically mild outbreak in commercial turkey breeders has shown promise as an efficacious and safe live vaccine in poultry in previous studies (8, 9).

The important characteristics of an ideal live MG vaccine, aside from safety in the target animals and efficacy, include 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). The vaccine should not spread to neighboring flocks (31).

The duration of immunity elicited by a live vaccine may be associated with the colonization and persistence of the vaccine in the respiratory tract. 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, 24).

The transmissibility of live MG vaccines is an important characteristic in determining the likelihood of the strain spreading to unvaccinated neighboring chickens and turkeys. F strain is readily transmissible to unvaccinated pen mates and chickens in adjacent pens (14). It can be isolated from farms long after vaccination has ceased and has been implicated in MG outbreaks in commercial turkeys (22). Although experimentally F strain has been shown to transmit from bird to bird, the widespread use of the vaccine has not resulted in widespread isolations of F strain from field cases in chickens (10).

The ts-11 and 6/85 vaccines have both been shown to be poorly transmissible to in contact poultry (23, 33).

Although 6/85 has been reported not to persist in the trachea and to be poorly transmissible, a "6/85-like" isolate was isolated from vaccinated and unvaccinated contact chickens long after vaccination (29).

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 (31). Experimental passage of 6/85 through chickens and turkeys did not result in a substantial increase in virulence (5, 6). Attempts to serially passage ts-11 in birds were unsuccessful but the vaccine appeared to retain its characteristics after three passages (33).

In this study we conducted three trials to further characterize K5054 with respect to stability by *in vivo* passages through chickens (Trial 1); persistence and the duration of

immunity elicited by a single vaccination (Trial 2); and transmissibility to unvaccinated chickens (Trial 3).

Materials and Methods

MG strains and isolates. K5054 was isolated from sinus exudate of a commercial turkey flock. This flock did not exhibit typical clinical signs of MG infection (8). R strain is a well-characterized virulent MG strain (28).

Serology. Sera were analyzed for MG antibodies using the serum plate agglutination (SPA) test using commercial antigen (Intervet America, Millsboro, Del), and the hemagglutination-inhibition (HI) test with antigen prepared from A5969 strain and chicken erythrocytes.

The SPA and HI test were carried out according to procedures described by Kleven (16). An SPA score $\geq$ 1 was considered positive. An HI titer of 1:20 was considered suspect and $\geq$ 1:40 was considered positive.

Commercial enzyme-linked immunosorbent assays (ELISA) were also performed on the sera (IDEXX, Westbrook, Maine).

Isolation and identification of mycoplasma. Cotton swabs from trachea and air sacs were used for culture. They were inoculated in Frey's modified broth and agar and incubated at 37 C. Mycoplasma isolates were identified using direct immunofluorescence (16).

Polymerase chain reaction (PCR). MG PCR was performed on pooled samples of tracheal swabs in Trial 3 at 12 weeks post vaccination/challenge. The PCR was carried out with modified primers and procedure described by Lauerman *et al.* (8, 19)

Random amplified polymorphic DNA (RAPD) analysis. MG isolates were characterized by RAPD analysis. The procedure and primers used were described by Fan *et al*.(7). A minimum of 20% of isolates recovered from vaccinated and challenged chickens were typed by this method.

DNA sequence analysis. DNA sequences of chicken-passaged isolates in Trial 1 and reference strains were compared as previously described (8). The sequences of the *pvpA* gene (GenBank accession number AF224059) (3) as described by Liu *et al.* (25) using a polymerase chain reaction with primers 3R and 4F (given as *pvpA* 1F and *pvpA* 2R). DNA sequences from a PCR product from a lipoprotein sequence (LP) described by Nascimento *et al.* (27), GenBank accession number AF075588, using primers described (8) were also compared. Sequences from the *mgc1* gene (13), GenBank accession number U34842, using described primers (8) were compared. This gene is analogous to a gene known as *gapA* (12). Sequence analysis was performed with MegAlign (DNASTAR, Lasergene, Inc. Madison, Wisconsin).

Evaluation of lesions. The lesions in chickens necropsied during the study were evaluated grossly by air sac lesions scoring on a scale from 0 to 4 (18). The tracheal lesions were evaluated microscopically by measuring the width of the tracheal mucosa. A section was collected from the upper third of the trachea (approximately 1 inch distal from the larynx) and fixed in 10% neutral formalin. The tracheal mucosa thickness was measured at four equidistant points on histological slides of cross sections of tracheas (31).

Chickens and Experimental Design. Trial 1. In the first part of Trial 1, fifteen layer-type male chickens (Hy-line, West Des Moines, Iowa) were obtained from a

commercial hatchery at one day of age. One week later and every week for 9 weeks, fifteen more chickens were obtained from the same hatchery. They were housed in Horsfal isolator units under positive pressure. The fifteen oldest chickens were inoculated with K5054 (8.4 x 10^8 color changing units (CCU)/ml) via eye drop at three weeks of age. One week later tracheal swabs from these chickens were used to inoculate 15 naive chickens (3 weeks of age) via the intratracheal route. This process was repeated until the isolate was passaged 10 times at 7-day intervals through chickens.

In the second part of this trial, one hundred and twenty layer-type male chickens were obtained from the hatchery at one day of age. These chickens were divided into 4 treatment groups and housed in four 3x3-m^2 colony houses with pine shaving litter. An isolate from the 10^{th} *in vivo* passage (K5383-2; 4.0 x10^7 CCU/ml) was used to challenge a group of 30 naïve chickens. Two other groups of 30 chickens were challenged with K5054 (2.3 x10^8 CCU/ml) or R strain (6.2 x10^8 CCU/ml). These challenges were administered by coarse spray (18) using a commercial paint sprayer (Preval® Sprayer Division, Precision Valve Corporation, Yonkers, NY); approximately 1ml of culture was administered per bird. The response to challenge was compared to that of 30 unchallenged negative controls. Ten days post challenge the chickens were bled for serology; swabs were taken of the trachea and air sacs for culture, the air sac lesions were scored and a section of trachea taken for histological examination.

Trial 2. One hundred and eighty layer-type male chickens (Hy-line, West Des Moines, Iowa) were acquired from a commercial hatchery at one day of age. Ninety of these chickens were housed in two floor pens (1.5x3 m^2) with pine shaving litter. The remaining ninety chickens were divided in 3 groups of thirty chickens and housed in

three 3x3-m^2 colony houses with pine shaving litter. The ninety chickens in the colony houses were vaccinated with K5054 (8.42 x 10^8 CCU/ml) via coarse spray at 3 weeks of age. At 4, 8 12, 16 and 28 weeks post vaccination 10 vaccinated birds from the colony houses and 10 unvaccinated birds from the floor pens (positive controls) were moved into two different colony houses (3x3-m^2) for challenge with virulent R strain. Five unvaccinated and unchallenged chickens served as negative controls. The titers of the R strain used in each of the challenges were 6.2 x 10^8 CCU/ml, 1.1 x 10^9 CCU/ml, 1.2 x 10^9 CCU/ml, 1.0 x 10^8 CCU/ml and 4.7 x 10^8 CCU/ml, respectively. Ten days post challenge the chickens were evaluated as in Trial 1. At 4, 8, 12, 16 and 28 weeks post vaccination 10 vaccinated (unchallenged) chickens were also bled for serology and cultured for mycoplasma. At the end of the experiment remaining chickens were euthanized.

Trial 3. One hundred and eighty layer-type female chickens (Hy-line, West Des Moines, Iowa) were acquired from a commercial hatchery at one day of age. These birds were housed in two floor pens (1.5x3 m^2) with pine shaving litter until at approximately 5 weeks of age they were divided into 6 treatment groups of 30 and moved to 6 different floor pens (1.5x3 m^2) with pine shaving litter.

Five chickens in one group were inoculated with K5054 (4.7 x 10^8 CCU/ml) via eye drop. These chickens (seeders) were placed in a pen with 25 naïve chickens (direct contacts), 30 chickens were placed in a pen immediately adjacent to the seeders and direct contacts (across-wire contacts). Another group of 30 chickens were placed in a pen separated from the direct contacts and seeders by an empty pen (across-empty-pen contacts). Five chickens were also inoculated with R strain (2.4 x 10^8 CCU/ml) and placed with direct contacts, across-wire contacts and across-empty-pen contacts in a

similar configuration. Five chickens from each of the 6 groups were bled for serology and tracheal swabs were obtained for mycoplasma culture at 1, 2, 4 and 8 weeks post inoculation of the seeders. At 12 weeks post inoculation all of the birds were bled, cultured and subsequently euthanized.

Chickens that died during the course of the trials were examined for gross lesions associated with MG.

The chickens in these trials were euthanized by cervical dislocation or with carbon dioxide according to the animal care and use policies of The University of Georgia.

Statistical analysis. Air sac lesion scores were analyzed using the Kruskal-Wallis Rank Sums test. The mean tracheal mucosa thickness was analyzed using the Tukey-Kramer HSD test. JMP® Statistics Made Visual (SAS Institute Inc., SAS Campus Drive, Cary, NC 27513). A P value $\leq$ 0.05 was considered significant.

Results

Trial 1. The results of this experiment are summarized in Tables 4.1 and 4.2. The air sac lesion scores and tracheal mucosa measurements of the K5383-2 (bird-passaged) group were not significantly different from the K5054 or negative control groups ($P \leq$ 0.05). The mean agglutination grade on the SPA of the K5383-2 group was higher than that of the K5054 group. There was also a higher proportion of MG isolations from the air sacs of the group challenged with K5383-2. The chicken-passaged isolates form the first, fifth and tenth passages had RAPD patterns that were identical to the K5054 strain.

DNA sequence analysis also showed that the DNA sequence of *pvpa*, *mgc1* and LP were 100% similar to K5054.

Trial 2. The results of this trial are summarized in Tables 4.3, 4.4 and 4.5. The K5054 strain persisted in the tracheas of vaccinated birds; it was isolated from tracheal swabs up to 28 weeks post-vaccination (Table 4.3). The proportion of tracheal samples from which MG was isolated declined to 6/9 (67%) at 16 weeks post vaccination and 5/10 (50%) at 28 weeks post vaccination. MG antibodies could be detected in the vaccinated chickens by SPA and ELISA up to 28 weeks post vaccination.

The serological responses of chickens vaccinated with K5054 and challenged with R strain at 4, 8 12, 16 or 28 weeks postvaccination are summarized in Table 4.4. The chickens vaccinated with K5054 and challenged with R strain did not have lesion scores that were significantly different from the negative controls in any of the five challenges during the trial ($P \leq 0.05$) (Table 4.5).

Trial 3. The results of this trial are summarized in Tables 4.6 and 4.7. The R strain spread to the direct contacts and could be detected by culture 2 weeks post challenge. At 12 weeks postchallenge R strain was isolated from all of the seeders and 23 of 25 (92%) penmates. This strain did not spread to chickens separated from the seeders by a wire fence or an empty pen.

K5054 failed to transmit to any of the in contact groups for the duration of the experiment but was reisolated from all of the seeders at 12 weeks post vaccination.

Discussion

Previous studies have shown that K5054 is a safe and efficacious vaccine in layer-type chickens and turkeys. To be an effective live MG vaccine K5054 must also have a low rate of transmission to unvaccinated poultry, be stable and consistently avirulent, and provide long lasting immunity.

K5054 did not transmit to in contact chickens in Trial 3 although it was re-isolated from all of the vaccinated birds (seeders). The SPA tests that were weakly positive at 8 weeks postvaccination were likely due to non-specific reactions (2, 4, 11). These positives were unconfirmed by the HI or ELISA tests and disappeared in the subsequent samplings.

R strain was transmitted to many of the direct contacts (92%) although it did not spread to the indirect contacts. In our experience with R strain, it sometimes spreads very well to contacts and sometimes it does not. The conditions of this study may not have been ideal for the transmission of MG. The transmission of MG strains may be variable and unpredictable from study to study. Whithear et. al., was unable to show transmission of ts-11 to contacts (33) although in a different study Ley et. al., showed transmission of ts-11 to 0-40% of commingled pullets (23).

It must be noted that K5054 did spread, albeit very slowly, among the commercial turkeys from which it was originally isolated (8), although it did not spread experimentally between the chickens in this study or the turkeys in a previous study (8). A greater challenge to the seeders or a higher proportion of seeders may eventually allow the transmission of K5054 to contacts. It can be concluded however, that K5054 has a relatively low rate of transmission.

K5054 did not increase in virulence after ten passages in chickens and was also genetically stable; there was no change in the RAPD pattern or DNA sequence of selected genes.

We believe that a naturally occurring MG strain of low virulence, such as K5054, may have the advantage of increased stability over many *in vivo* passages as compared to laboratory attenuated strains. F strain is likely to have originated from a naturally occurring strain of moderate virulence and there is no published evidence of an increase in virulence although it has been used for many years. However, the possibility of "escape" and some increase in the level of virulence cannot be ignored with any live mycoplasma vaccine in poultry production situations. Diligence should always be applied when using a live vaccine; which can spread, multiply and change its characteristics when given the opportunity, however small the possibility.

A vaccine strain should persist and multiply in the trachea long enough to elicit a protective immune response (31). The duration of immunity elicited may be associated with the colonization and persistence of the vaccine in the respiratory tract. In this study K5054 persisted in the upper respiratory tract and elicited protective immunity for the duration of the study (7 months). With respect to persistence K5054 seems to be comparable to F strain and ts-11.

To conclude K5054 appears to be not only a safe and efficacious vaccine but also to be stable following ten *in vivo* passages, it has a low rate of transmission, the vaccine persists in the upper respiratory tract for long periods, and a single vaccination results in long lasting immunity. Further study is needed to evaluate the amenability of this strain to commercial production and application.

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Table 4.1. Trial 1 (Stability). Serological response of chickens 10 days postchallenge with R strain, K5054 or K5383-2 (isolate from 10th *in vivo* passage of K5054).

Challenge	SPA	HI	ELISA
R strain	30/30 [A] (4.0)[B]	2/30 (4.2) [C]	26/30 (0.9 ± 0.5) [D]
K5054	0/30 (0.0)	0/30 (0.0)	0/30 (0.1 ± 0.0)
K5383-2	27/30 (1.6)	0/30 (0.0)	3/30 (0.1 ± 0.3)
None	0/30 (0.0)	0/30 (0.0)	0/30 (0.1 ± 0.0)

[A] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, and ELISA: ≥ 0.6)

[B] Mean agglutination grade (from 0 to 4).

[C] Geometric mean titer

[D] Geometric mean sample/positive ratio ± standard deviation

Table 4.2. Trial 1 (Stability). Lesion scores and MG isolation from chickens 10 days post challenge with R strain, K5054 or K5383-2 (isolate from 10th *in vivo* passage of K5054) [A].

Challenge	Air sac lesion score[B]	Tracheal mucosal thickness[C]	MG isolation[D]	
			Trachea	Air sacs
R strain	27/30[E] (2.0 ± 1.2)[Fa]	32.27 ± 14.30[a]	30/30[a]	30/30[a]
K5054	0/30 (0.0 ± 0.0) [b]	5.88 ± 1.04[b]	29/29[a]	4/30[b]
K5383-2	6/30 (0.2 ± 0.5) [b]	6.32 ± 2.05 [b]	30/30[a]	23/30[c]
None	0/30 (0.0 ± 0.0) [b]	6.63 ± 1.49 [b]	0/30[b]	0/30[d]

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] Macroscopically scored from 0 to 4

[C] Mean thickness for the group in units. 1 unit = 0.0083mm

[D] MG isolates from group challenged with R strain were identified as R strain by RAPD analysis; MG isolates from groups challenged with K5054 or K5383-2 were identified as similar to K5054 by RAPD analysis.

[E] No. of positive samples/No. of tested samples (Air sac score ≥ 1)

[F] Mean score ± SD

Table 4.3. Trial 2 (Persistence). Serological response and MG isolation from the tracheas of chickens post vaccination with K5054.

Wk post vaccination	SPA	HI	ELISA	MG isolation[A]
4	9/10[B] (1.6)[C]	0/10 (0.0)[D]	1/10 (0.1 $\pm$ 0.2)[E]	10/10
8	10/10 (3.5)	0/10 (3.3)	10/10 (1.6 $\pm$ 0.4)	9/9
12	10/10 (4.0)	0/10 (0.0)	10/10 (2.3 $\pm$ 0.8)	8/8
16	10/10 (4.0)	0/10 (0.0)	10/10 (1.3 $\pm$ 0.6)	6/9
28	10/10 (2.7)	4/10 (26.4)	6/10 (0.8 $\pm$ 1.0)	5/10

[A] Recovered MG isolates were all identified as K5054 by RAPD analysis.

[B] No. of positive samples/No. of tested samples (SPA: $\geq$1, HI: $\geq$40, and ELISA: $\geq$ 0.6)

[C] Mean agglutination grade (from 0 to 4).

[D] Geometric mean titer

[E] Geometric mean sample/positive ratio $\pm$ standard deviation

Table 4.4. Trial 2 (Duration of Immunity). Serological response of chickens vaccinated with K5054 and 10 days post challenge with R strain.

Wk PV	Group	Challenged	SPA	HI	ELISA
	K5054	Yes	10/10[A] (4.0)[B]	0/10 (1.4)[C]	5/10 (0.6 ± 0.6)[D]
4	No Vaccine	Yes	10/10 (4.0)	2/10 (8.6)	4/10 (0.4 ± 0.3)
	No Vaccine	No	0/5 (0.0)	0/5 (0.0)	0/5 (0.0 ± 0.0)
	K5054	Yes	10/10 (4.0)	2/10 (6.9)	10/10 (2.7 ± 1.1)
8	No vaccine	Yes	10/10 (4.0)	1/10 (2.0)	10/10 (1.0 ± 0.5)
	No vaccine	No	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.0)
	K5054	Yes	10/10 (4.0)	1/10 (4.8)	8/10 (1.2 ± 0.9)
12	No vaccine	Yes	10/10(3.8)	0/10 (2.5)	3/10 (0.4 ± 0.5)
	No vaccine	No	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.0)
	K5054	Yes	10/10 (4.0)	4/10 (6.3)	10/10 (3.9 ± 1.6)
16	No vaccine	Yes	10/10 (3.0)	0/10 (0.0)	10/10 (1.0 ± 0.3)
	No vaccine	No	0/5 (0.0)	0/5 (0.0)	0/5 (0.2 ± 0.0)
	K5054	Yes	10/10 (4.0)	10/10 (60.6)	10/10 (2.4 ± 1.8)
28	No vaccine	Yes	10/10 (2.4)	8/10 (29.7)	5/10 (0.4 ± 0.8)
	No vaccine	No	0/5 (0.0)	0/5 (0.0)	0/5 (0.2 ± 0.2)

[A] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, and ELISA: ≥ 0.6)

[B] Mean agglutination grade (from 0 to 4).

[C] Geometric mean titer

[D] Geometric mean sample/positive ratio ± standard deviation

Table 4.5. Trial 2 (Duration of Immunity). Lesion scores and MG isolation from K5054-vaccinated chickens 10 days post challenge with R strain [A].

Wk PV	Group	Challenged	Air sac lesion score[B]	Tracheal mucosal thickness[C]	MG isolation[D] Trachea	MG isolation[D] Air sac
4	K5054	Yes	4/10[E] (0.4 ± 0.5)[Fa]	9.45 ± 3.21[a]	10/10[a]	8/10[a]
	No Vaccine	Yes	10/10 (3.2 ± 0.8)[b]	25.44 ± 10.67[b]	10/10[a]	10/10[a]
	No Vaccine	No	0/5 (0.0 ± 0.0)[a]	10.35 ± 2.23[a]	0/5[b]	0/5[b]
8	K5054	Yes	1/10 (0.1 ± 0.3) [a]	11.85 ± 4.31[a]	10/10[a]	5/10[a]
	No vaccine	Yes	10/10 (2.3 ± 0.8)[b]	28.53 ± 15.49[b]	10/10[a]	10/10[b]
	No vaccine	No	0/5 (0.0 ± 0.0)[a]	10.05 ± 1.19[a]	0/5[b]	0/5[c]
12	K5054	Yes	0/10 (0.0 ± 0.0)[a]	10.70 ± 1.38[a]	10/10 [a]	10/10 [a]
	No vaccine	Yes	10/10(2.5 ± 0.7)[b]	20.22 ± 5.45[b]	8/8 [a]	10/10 [a]
	No vaccine	No	0/5 (0.0 ± 0.0)[a]	10.73 ± 1.20[a]	0/5 [b]	0/5 [b]
16	K5054	Yes	6/10 (0.9 ± 0.9)[a]	12.97± 2.36[a]	6/6[a]	7/9[a]
	No vaccine	Yes	10/10 (3.0 ± 0.0)[b]	34.82 ± 17.04[b]	10/10[a]	9/10[a]
	No vaccine	No	0/5 (0.0 ± 0.0)[c]	12.95 ± 1.41[a]	0/5[b]	0/5[b]
28	K5054	Yes	7/10 (0.8 ± 0.6)[a]	13.83 ± 3.14[a]	9/10[a]	9/10[a]
	No vaccine	Yes	10/10 (3.3 ± 0.7)[b]	36.18 ± 16.44[b]	10/10[a]	10/10[a]
	No vaccine	No	0/5 (0.0 ± 0.0)[c]	13.56 ± 3.65[a]	0/10[b]	0/10[b]

[A] Values within a column with a different lower case superscript are significantly different ($P \leq 0.05$)

[B] Macroscopically scored from 0 to 4

[C] Mean thickness for the group in units. 1 unit = 0.0083mm

[D] MG isolates recovered from groups challenged with R strain were all identified as R strain by RAPD analysis.

[E] No. of positive samples/No. of tested samples (Air sac score $\geq$ 1)

[F] Mean score $\pm$ SD

Table 4.6. Trial 3 (Transmissibility). Serological response and MG isolation from the tracheas of chickens postvaccination with K5054 by eye drop (seeders), direct contact, indirect contact between neighboring pens (adjacent) or with an empty pen separating.

Weeks PC	K5054 Contact	SPA	HI	ELISA	MG isolation
1	Direct	2/5 [A] (0.4)[B]	0/5 (0.0) [C]	0/5 (0.1 ± 0.0) [D]	0/5
	Adjacent	2/5 (0.4)	0/5 (0.0)	0/5 (0.0 ± 0.0)	0/5
	Empty pen	3/5 (0.6)	0/5 (0.0)	0/5 (0.1 ± 0.1)	0/5
2	Direct	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.0)	0/5
	Adjacent	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.0)	0/5
	Empty pen	0/5 (0.0)	0/5 (0.0)	0/5 (0.2 ± 0.0)	0/5
4	Direct	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.0)	0/5
	Adjacent	0/5 (0.0)	0/5 (0.0)	0/5 (0.2 ± 0.1)	0/5
	Empty pen	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.1)	0/5
8	Direct	5/5 (1.6)	0/5 (0.0)	0/5 (0.1 ± 0.0)	0/5
	Adjacent	5/5 (2.2)	0/5 (0.0)	0/5 (0.0 ± 0.0)	0/5
	Empty pen	5/5 (1.2)	0/5 (0.0)	0/5 (0.1 ± 0.1)	0/5
12	Seeders	5/5 (3.4)	0/5 (0.0)	0/5 (0.1 ± 0.1)	5/5
	Direct	0/25 (0.0)	0/25 (0.0)	0/25 (0.1 ± 0.1)	0/25
	Adjacent	0/30 (0.0)	0/30 (0.0)	2/30 (0.1 ± 0.3)	0/30

Empty pen	0/26 (0.0)	0/26 (0.0)	0/26 (0.1 ± 0.1)	0/26

[A] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥40, and ELISA: ≥ 0.6)

[B] Mean agglutination grade (from 0 to 4).

[C] Geometric mean titer

[D] Geometric mean sample/positive ratio ± standard deviation

Table 4.7. Trial 3 (Transmissibility). Serological response and MG isolation from the tracheas of chickens post challenge with R strain by eye drop (seeders), direct contact, indirect contact between neighboring pens (adjacent) or with an empty pen separating.

Wk PC	R strain Contact	SPA	HI	ELISA	MG isolation[A]
1	Direct	0/5[B] (0.0)[C]	0/5 (0.0)[D]	0/5 (0.0 ± 0.0)[E]	0/5
	Adjacent	1/5 (0.2)	0/5 (0.0)	0/5 (0.0 ± 0.1)	0/5
	Empty pen	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.0)	0/5
2	Direct	0/5 (0.0)	0/5 (0.0)	0/5 (0.0 ± 0.0)	3/5
	Adjacent	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.0)	0/5
	Empty pen	0/5 (0.0)	0/5 (0.0)	0/5 (0.1 ± 0.0)	0/5
4	Direct	3/5 (2.2)	0/5 (0.0)	0/5 (0.2 ± 0.1)	5/5
	Adjacent	0/5 (0.0)	0/5 (0.0)	0/5 (0.2 ± 0.0)	0/5
	Empty pen	0/5 (0.0)	0/5 (0.0)	1/5 (0.1 ± 0.2)	0/5
8	Direct	5/5 (3.6)	0/5 (0.0)	0/5 (0.2 ± 0.1)	5/5
	Adjacent	4/5 (2.4)	0/5 (0.0)	0/5 (0.1 ± 0.0)	0/5
	Empty pen	4/5 (1.4)	0/5 (0.0)	0/5 (0.1 ± 0.1)	0/5
12	Seeders	5/5 (4.0)	5/5 (69.6)	5/5 (2.1 ± 0.6)	5/5
	Direct	25/25 (4.0)	17/25 (35.1)	25/25 (1.3 ± 0.6)	23/25
	Adjacent	0/30 (0.0)	4/30 (2.4)	0/30 (0.0 ± 0.1)	0/30
	Empty pen	0/30 (0.0)	0/30 (0.0)	0/30 (0.1 ± 0.1)	0/30

[A] MG isolates recovered from the seeders or groups in contact with R strain or K5054 seeders were identified as R strain or K5054 respectively by RAPD analysis.

[B] No. of positive samples/No. of tested samples (SPA: ≥1, HI: ≥ 40, and ELISA: ≥ 0.6)

[C] Mean agglutination grade (from 0 to 4).

[D] Geometric mean titer

[E] Geometric mean sample/positive ratio ± standard deviation

CHAPTER 5

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS AND DNA SEQUENCE ANALYSIS OF THREE GENES IN THE MOLECULAR EPIDEMIOLOGY OF MYCOPLASMA GALLISEPTICUM[4]

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Key words: *Mycoplasma gallisepticum*, random amplified polymorphic DNA

Abbreviations: RAPD = random amplified polymorphic DNA; PCR= polymerase chain reaction

Summary

Mycoplasma gallisepticum isolates from the USA, Israel and Australia were characterized by random amplified polymorphic DNA (RAPD) as well as DNA sequence analysis of portions of the phase-variable putative adhesin protein (*pvpA*) gene, the cytadhesin *gapA* gene and an uncharacterized lipoprotein (LP) sequence. The results were compared to reference strains (vaccine and laboratory strains). Sequence analysis of any one of the genes did not result in definitive identification of isolates. However, by combining the data from DNA sequencing of all three genes, patterns could be identified in recognizing related isolates and unique strains. The RAPD analysis and combined DNA sequence analysis data correlated well. The Australian isolates appeared to be more similar to the US isolates than were the Israeli isolates. It appeared that the LP and *gapA* genes are more highly conserved than *pvpA* gene.

Introduction

Control of *Mycoplasma gallisepticum* 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. Serological monitoring performed periodically and isolation of *M. gallisepticum* or DNA based detection methods used to confirm the presence of *M. gallisepticum* (16, 19).

One of the options for control of *M. gallisepticum* in situations where complete eradication is difficult to attain is live vaccines (13, 30). Three live *M. gallisepticum* vaccines are currently used worldwide, they are: F-strain (Schering Plough, Kenilworth, N.J.), ts-11 (Bioproperties, Inc., Australia, marketed in the US by Merial select Laboratories, Gainsville, GA.) and 6/85 (Intervet America, Millsboro, Del.).

In recent years, a reemergence of mycoplasma infection has necessitated a reevaluation of control strategies for *M. gallisepticum* and increased the need to differentiate between vaccine strains and field isolates (13).

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 (25).

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 (28).

Molecular techniques that have been used to identify *M. gallisepticum* strains include restriction fragment length polymorphisms (RFLP) of DNA (12, 14), DNA and ribosomal RNA gene probes (11, 34), and PCR with strain-specific primers (24).

The most widely used method for differentiating *M. gallisepticum* strains is random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR, analysis (3, 5, 6). The RAPD assay was first described by Williams et al. (33) and Welsh and McClelland (29). RAPD analysis is quick and sensitive and this method has been used to identify vaccine strains in *M. gallisepticum* -vaccinated flocks and for epidemiological studies (10, 15, 17, 18).

However, 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 *M. gallisepticum* subtype may be present. Furthermore, technical factors such as target DNA/primer ratio may significantly impact the reproducibility of RAPD patterns. The amplification process of the RAPD reaction is extremely sensitive to slight changes in the annealing temperature; this can also lead to variability in the banding patterns. The use of empirically designed primers, each with its own optimal reaction conditions and reagents, also makes standardization of the technique difficult.

Ultimately, all molecular genetic methods for distinguishing organism subtypes are based on differences in the DNA sequence. DNA sequencing would therefore appear to be an appropriate approach to differentiating subtypes.

Progress in the molecular biology of mycoplasmas has resulted in the description of several surface proteins in virulent mycoplasmas, many of them cytadhesins including pMGA (22), PvpA (35), MGC1 (7, 9) and MGC2 (8). Adhesion is prerequisite for colonization and for infection. Loss of adhesion by mutation results in loss of infectivity, reversion to cytadherence phenotype accompanied by regaining infectivity and virulence (26).

PvpA is postulated to be one of the accessory membrane proteins involved in cytadherence of *M. gallisepticum*. Variation within PvpA could affect the specificity or affinity of adherence. It is an integral membrane surface protein with a free C terminus that is subject to spontaneous high-frequency phase variation in expression and exhibits size variation among strains (2, 35). Deletions within the 3' end of the *pvpA* gene results in size variation of the PvpA polypeptide from 48 to 55 kDa. The deletions were localized at the proline-rich carboxy-terminal region and within two direct repeat sequences (2), this domain may be under selective pressure in the host. Several *M. gallisepticum* strains differing in their adherence and pathogenicity have varying deletions and sizes of PvpA. Analysis of *pvpA* has been used to differentiate between *M. gallisepticum* strains (20).

The *gapA* gene (7), also know as *mgc1* (9), is one of three clustered genes. The two associated genes are *mgc2* and *mgc3* (also known as *crmA*). The *gapA* gene encodes a 105-kDa protein with homology to the P1 cytadhesin protein of *M. pneumoniae*. Immunoblot analysis of various strains has demonstrated intraspecies variation in the size of the GapA protein (98, 105 and 110 kDa) (7).

The LP nucleotide sequence analyzed in this paper was first described by Nasciemto et al. in a diagnostic PCR for *M. gallisepticum* (23). The open reading frame is thought to code for an uncharacterized lipoprotein.

In this study, reference strains and isolates form the USA, Israel and Australia were analyzed by RAPD analysis as well as by comparison of nucleotide sequence data of the *pvpA* and *gapA* genes as well as LP sequences. The results of RAPD analysis were compared to that of DNA sequencing.

Materials and Methods

Mycoplasma isolates. Sixty-four *M. gallisepticum* isolates were analyzed; there were 52 from the USA, 4 from Australia and 8 from Israel. The isolates from the USA were obtained from the depository at the Poultry Diagnostic and Research Center (PDRC) in Athens, GA. These isolates were from 16 different states and ranged from 1975 to 2001. The isolates were from broiler breeders, commercial layers, turkey breeders, meat-type turkeys, house finches and an American goldfinch. The Israeli isolates were obtained through the Division of Avian and Aquatic Diseases, Kimron Veterinary Institute, Bet Dagan 50250, Israel. Most of these isolates are from outbreaks that had no specific connection. The isolates were from broiler breeders, turkey breeders and meat-type turkeys. The Australian isolates were acquired courtesy of K.G. Whithear, the University of Melbourne, Australia. The origins of the isolates are listed in Table 5.1.

The reference strains used included vaccine strains such as F strain (1, 21), 6/85 (4) and ts-11 (31, 32). Laboratory strains such as HF-51 (a house finch isolate) R strain (27), A5969, and S6 (36) were also included.

Random amplified polymorphic DNA (RAPD) analysis. The procedure and primers used for RAPD analysis were described by Fan *et al.* (5). RAPD analysis was also conducted using primers described by Geary *et al.* (6) and Charlton *et al.* (3). Isolates that appeared identical with Fan's primers were then tested with the primers described by Geary and Charlton. Isolates were considered identical when major band differences could not be visualized with any of the three primer sets.

PCR and sequence analysis. DNA base sequences of the *pvpA* gene (GenBank accession number AF224059) (2) of the isolates and reference strains were compared as described by Liu *et al.* (20) using a polymerase chain reaction with primers 3R and 4F (given as *pvpA* 1F and *pvpA* 2R). DNA sequences from a PCR product from a lipoprotein gene (LP) described by Nascimento *et al.* (23), GenBank accession number AF075588, using primers LP-1F (GGA TCC CAT CTC GAC CAC GAG AAA A) and LP-2R (CTT TCA ATC AGT GAG TAA CTG ATG A) were also compared. Sequences corresponding to positions 30-541 were analyzed. Finally, sequences from the *mgc1* gene (9), GenBank accession number U34842, using primers Adhs1-3F (TTC TAG CGC TTT ARC CCT AAA CCC) and Adhs1-4R (CTT GTG GAA CAG CAA CGT ATT CGC) were compared. Positions 3823-4154 were compared. This gene is analogous to a gene known as *gapA* (7). The amplified products of these PCR's were sequenced at the Molecular Genetics Instrumentation Facility (MGIF), University of Georgia. Sequence analysis was performed with MegAlign (DNASTAR, Lasergene, Inc. Madison, Wisconsin).

RESULTS

RAPD analysis. RAPD analysis using 3 different primer sets resulted in a relatively high level discrimination between *M. gallisepticum* strains (Fig 5.1). One primer set did not always elucidate differences between *M. gallisepticum* strains; two isolates may have appeared very similar with one set of primers and definitely different with another set of primers. We therefore first conducted RAPD's using Fan's primers (5); if there was significant similarity between the patterns generated we then conducted RAPD's with primers described by Geary *et al.* (6) and Charlton *et al.* (3) to confirm or dispute the similarity between strains.

RAPD analysis identified 32 different patterns (strains) when the 64 isolates were analyzed. The patterns were designated A to AH. (Table 5.1).

DNA sequence analysis. These results are summarized and compared to the RAPD data in Table 5.2. The *pvpA* gene sequence analysis exhibited the most variability in nucleotide sequence differences among the strains (66.1 – 100% range of identities). The LP and *gapA* sequence analysis resulted in the less differentiation between the strains (95.1– 100%) and (94.3– 100%) range of identities respectively.

The reference strains S6 and HF-51 are 100% identical to each other with respect to the LP sequence. The reference strains 6/85, ts-11 and HF-51 are 100% identical to each other with respect to the *gapA* sequence.

Analysis of the combined sequence of all three genes allowed further discrimination between *M. gallisepticum* strains and correlated better with RAPD analysis than comparison of single DNA sequence data.

Eleven of 52 (21%) of the US field isolates were characterized as very similar to vaccine strains by RAPD and DNA sequence analysis. The sequence identities ranged from 98.8 – 100 % for each of the three DNA sequences.

The isolates that had RAPD patterns different from the reference strains (RAPD types E to AG) ranged in sequence identity to the reference strains from 93.4% to 100% for each of the 3 sequences analyzed.

Forty-one of 52 (79%) of the US field isolates were characterized as different from the reference strains. These isolates could be grouped within outbreaks by RAPD analysis or the combined sequence analysis data. 23 of these 41 isolates (56%) were similar to HF-51 with respect to the *pvpA* sequence (98.8 – 99.5% identity). Thirteen of these isolates also had LP (98.8-100% identity) and gapA (100% identity) sequences that were similar to HF-51.

There were some isolates that were very similar to one reference strain sequence and very similar to a different reference strain when a different region was compared. For example, the isolates that generated RAPD type I were 100% similar to R strain with respect to *pvpA* and 100% identical to 6/85, ts-11 and HF-51 with respect to the *gapA* sequence.

The isolates from house finches appeared to be closely related to each other with the exception of K4409. The *pvpA* gene of this isolate has a relatively large deletion of 170 nucleotides when compared to the other house finch isolates. K4409 however generates a RAPD pattern (D) indistinguishable from that of the other isolates.

Some isolates generated similar RAPD patterns although the DNA sequence data showed differences in the nucleotide sequences. These were isolates with RAPD patterns

designated A, E and H. Two isolates with RAPD type A have 4 nucleotide differences from the other isolates in the *gapA* sequence ($T_7 \rightarrow C_7$; $A_{16} \rightarrow C_{16}$; $C_{320} \rightarrow G_{320}$; and $C_{328} \rightarrow A_{328}$). The A pattern is generated by the F strain vaccine.

Two isolates with the RAPD type E had a single nucleotide substitution in the *gapA* sequence ($C_{328} \rightarrow A_{328}$) that was different from the other isolates in this group.

One isolate with the RAPD type H had a single nucleotide substitution in the *pvpA* sequence ($T_{375} \rightarrow A_{375}$).

One isolate (K5120) generated a unique RAPD pattern (M) although on DNA sequencing of all three regions it was 100% identical to the isolates that generated a different RAPD type (L).

The RAPD types K and L were similar although not indistinguishable from the 6/85 vaccine type (B), and designated "6/85-like". On sequence analysis these isolates were more similar to HF-51, ts-11 or F strain (98.0 – 99.8% identity) than 6/85 when the *pvpA* and LP sequences were analyzed.

The Israeli isolates were characterized as relatively similar to each other but different from the reference strains, the US and Australian isolates by both RAPD and DNA sequence analysis. The isolates could be differentiated into 6 types (AB to AH), although not easily, by RAPD analysis with multiple primer sets. The sequence analysis consistently resulted in clustering of these isolates away from the US and Australian isolates. The sequence data correlated with the RAPD analysis.

The Australian isolates could be differentiated from each other the other isolates by both RAPD and sequence analysis. The isolates were more similar to the reference

strains and US isolates than the Israeli isolates. The sequence data for these isolates did not result in a cluster of Australian isolates as did the Israeli.

Discussion

The ultimate goal of researchers involved in *M. gallisepticum* strain differentiation and epidemiology is a method that will differentiate strains easily, rapidly and reproducibly from clinical samples.

Methods of strain differentiation must have high differentiation 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. It is especially important for the construction of reliable databases containing known strains within a species to which unknown organisms can be compared.

In this study, it appeared that the LP and *gapA* sequences are more highly conserved than *pvpA*, these results are similar to those of a previous study (20).

The RAPD analysis and combined DNA sequence analysis data correlated well, although there were some isolates that indistinguishable on RAPD analysis but DNA sequencing revealed small differences (1 to 4 nucleotide substitutions). There was also an isolate that could not be differentiated by the DNA sequence data but RAPD analysis. generated a unique pattern.

Some of the USA field isolates were very similar to vaccine strains (6/85, ts-11 and F strain) with respect to RAPD patterns and nucleotide sequences. The number of isolates that were similar to vaccine strains may not be a true representation of the *M.*

gallisepticum epidemiology in the US because vaccine-like isolates are more likely to be selected for further study thereby skewing the sample away from "wild-type" isolates.

Similar to the results from previous studies (17), the isolates from house finches were closely related to each other and different from the reference strains and other field isolates with respect to RAPD patterns and DNA sequences. There was one US isolate (K5054) from turkeys that was also very similar to the house finch strains.

Most of the US isolates were not very similar to any of the reference strains, however similarities among isolates within and between *M. gallisepticum* outbreaks could be identified.

The Australian isolates appeared to be more closely related to the US isolates than were the Israeli isolates.

RAPD analysis is rapid and sensitive method and although RAPD analysis can successfully differentiate between *M. gallisepticum* strains there are disadvantages to the method that make amplification and sequencing of specific genes an attractive alternative for differentiation. A sequence database allows the comparison of many seemingly unrelated isolates. This method would have greater reproducibility and be more easily standardized.

However, 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 and the variability within the selected sequence must be sufficient to differentiate different strains of a particular species.

In this study, analysis of any one sequence did not result in definitive identification of the isolates. However, when all three sequences were analyzed the results were more useful and closely approached the discriminatory power of RAPD analysis.

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Table 5.1. Origin and RAPD type of the isolates analyzed in this study.

Isolate	Species	State/Country of Isolation	Year of Isolation	RAPD type
K5104	Turkey	VA, USA	2001	A (F)
K4781A	Turkey	VA, USA	1999	A
K5058E	Turkey	VA, USA	2001	A
K3944	Turkey	NC, USA	1995	B (6/85)
K4029	Turkey	NE, USA	1995	B
K4043	Turkey	NE, USA	1995	B
K4421A	Turkey	MI, USA	1997	B
K4423	Turkey	MI, USA	1997	B
K4465	Turkey	OH, USA	1997	B
K4688	Chicken	NC, USA	1999	C (ts-11)
K5109	Turkey	PA, USA	2001	C
K3839	Housefinch	MD, USA	1994	D (HF-51)
K4013	Housefinch	PA, USA	1995	D
K4094	Housefinch	TN, USA	1996	D
K4366	American Goldfinch	SC, USA	1997	D
K4409	Housefinch	TX, USA	1997	D
K4593	Housefinch	MD, USA	1998	D
K5054	Turkey	IN, USA	2001	D
K4110A	Turkey	NC, USA	1996	E

K4110B	Turkey	NC, USA	1996	E
K4110F	Turkey	NC, USA	1996	E
K4158C	Turkey	MO, USA	1996	E
K4181B	Chicken	AR, USA	1996	E
K4649A	Turkey	CO, USA	1998	G
K4649B	Turkey	CO, USA	1998	G
K4669A	Turkey	CO, USA	1998	G
K5011	Turkey	MD, USA	2000	H
K5027B	Turkey	IN, USA	2000	H
K5033A	Turkey	IN, USA	2000	H
K5033F	Turkey	IN, USA	2000	H
K2101	Chicken	CO, USA	1994	I
K4385	Turkey	CO, USA	1997	I
K4414A	Turkey	CO, USA	1997	I
K4902	Turkey	CO, USA	2000	I
K4181C	Chicken	AR, USA	1996	J
K4246	Turkey	AR, USA	1996	J
K4280	Chicken	MO, USA	1996	J
K4311	Turkey	AR, USA	1996	J
K5029B	Turkey	PA, USA	2000	K
K5037A	Turkey	PA, USA	2000	K
K5039H	Turkey	PA, USA	2000	K
K4236	Turkey	VA, USA	1996	L

K5120	Turkey	GA, USA	2001	M
K4657	Chicken	GA, USA	1998	N
K4705	Chicken	AR, USA	1999	O
K4931	Turkey	VA, USA	2000	P
K435	Turkey	GA, USA	1973	Q
K503	Chicken	GA, USA	1974	S
K703	Chicken	MD, USA	1975	T
K730	Chicken	GA, USA	1975	U
K3020	Turkey	CA, USA	1990	V
K4355	Chicken	CA, USA	1996	W
97019 E3	Chicken	Australia	1997	X
94043 30-5a	Chicken	Australia	1994	Y
95002 16a	Chicken	Australia	1995	Z
96002 6-3a	Chicken	Australia	1996	AA
99169 2NSb	Chicken	Australia	1999	AB
BRT-14	Chicken	Israel	2000	AC
DSD-6	Turkey	Israel	2000	AC
KS-2	Chicken	Israel	2001	AD
KSC-3	Chicken	Israel	1999	AE
UHP-1	Chicken	Israel	1999	AF
YBS-2	Turkey	Israel	2000	AF
MK-8	Chicken	Israel	2001	AG
OR-2	Chicken	Israel	2001	AH

Table 5.2. Summary of the RAPD and DNA sequence analysis results.

RAPD Type[A]	No. of Isolates	*pvpA*[B] (467)[C]	LP (490)	*gapA* (332)
A (F)	3	F (100%)	F (100%)	F (98.8% -100%)
B (6/85)	6	6/85 (100%)	6/85 (99.8 - 100%)	6/85 (100%)
C (ts-11)	2	ts-11 (100%)	ts-11(100%)	ts-11(100%)
D (HF-51)	7	HF-51 (97.9 - 100%)	HF-51/S6 (100%)	HF-51 (100%)
E	5	HF-51 (99.5%)	HF-51/S6 (100%)	R (99.4%)
G	3	HF-51 (98.8%)	HF-51/S6 (100%)	A5969 (99.4%)
H	4	HF-51 (99.3 - 99.5%)	HF-51/S6 (99.0%)	R (99.7%)
I	4	R (100%)	F (98.0%)	6/85/ts-11/HF-51 (100%)
J	4	HF-51 (99.5%)	ts-11(100%)	6/85/ts-11/HF-51 (100%)
K	3	HF-51 (98.8%)	F (98.0%)	6/85/ts-11/HF-51 (100%)
L	1	HF-51 (99.5%)	ts-11(99.8%)	6/85/ts-11/HF-51 (100%)
M	1	HF-51 (99.5%)	ts-11(100%)	6/85/ts-11/HF-51 (100%)
N	1	F (98.7%)	6/85 (99.6%)	R (99.7%)
O	1	HF-51 (99.0%)	HF-51/S6 (99.8%)	A5969 (99.4%)
P	1	HF-51 (99.5%)	R (99.0%)	6/85/ts-11/HF-51 (100%)
Q	1	R (99.8%)	R (100%)	A5969 (99.4%)
S	1	F (89.0%)	HF-51/S6 (94.3%)	F (99.7%)
T	1	F (88.6%)	HF-51/S6 (94.1%)	F (98.8%)
U	1	F (89.0%)	6/85 (94.7%)	F (98.5%)

V	1	6/85 (97.5%)	R (100%)	R (99.7%)
W	1	S6 (100%)	HF-51/S6 (100%)	S6 (97.6%)
X[D]	1	F (99.2%)	ts-11 (98.6%)	6/85/ts-11/HF-51 (100%)
Y[D]	1	6/85 (99.3%)	HF-51/S6 (100%)	6/85/ts-11/HF-51 (100%)
Z[D]	1	ts-11 & R (98.1%)	ts-11 (99.8%)	6/85/ts-11/HF-51 (100%)
AA[D]	1	ts-11(98.5%)	HF-51/S6 (100%)	A5969 (99.1%)
AB[D]	1	ts-11(99.1%)	HF-51/S6 (100%)	6/85/ts-11/HF-51 (100%)
AC[E]	2	F (91.5%)	F (98.2%)	6/85/ts-11/HF-51 (96.4%)
AD[E]	1	F (91.1%)	F (98.2%)	6/85/ts-11/HF-51 (96.4%)
AE[E]	2	F (91.5%)	F (98.0%)	6/85/ts-11/HF-51 (96.4%)
AF[E]	1	F (91.5%)	F (98.0%)	6/85/ts-11/HF-51 & F (97.0%)
AG[E]	1	F (91.5%)	F (98.2%)	6/85/ts-11/HF-51 (96.4%)
AH[E]	1	F (91.5%)	F (98.2%)	6/85/ts-11/HF-51 (96.4%)

[A] Similar to reference strains (F, 6/85, ts-11, HF-51, R strain, A5969 or S6) or unique patterns (A to AH)

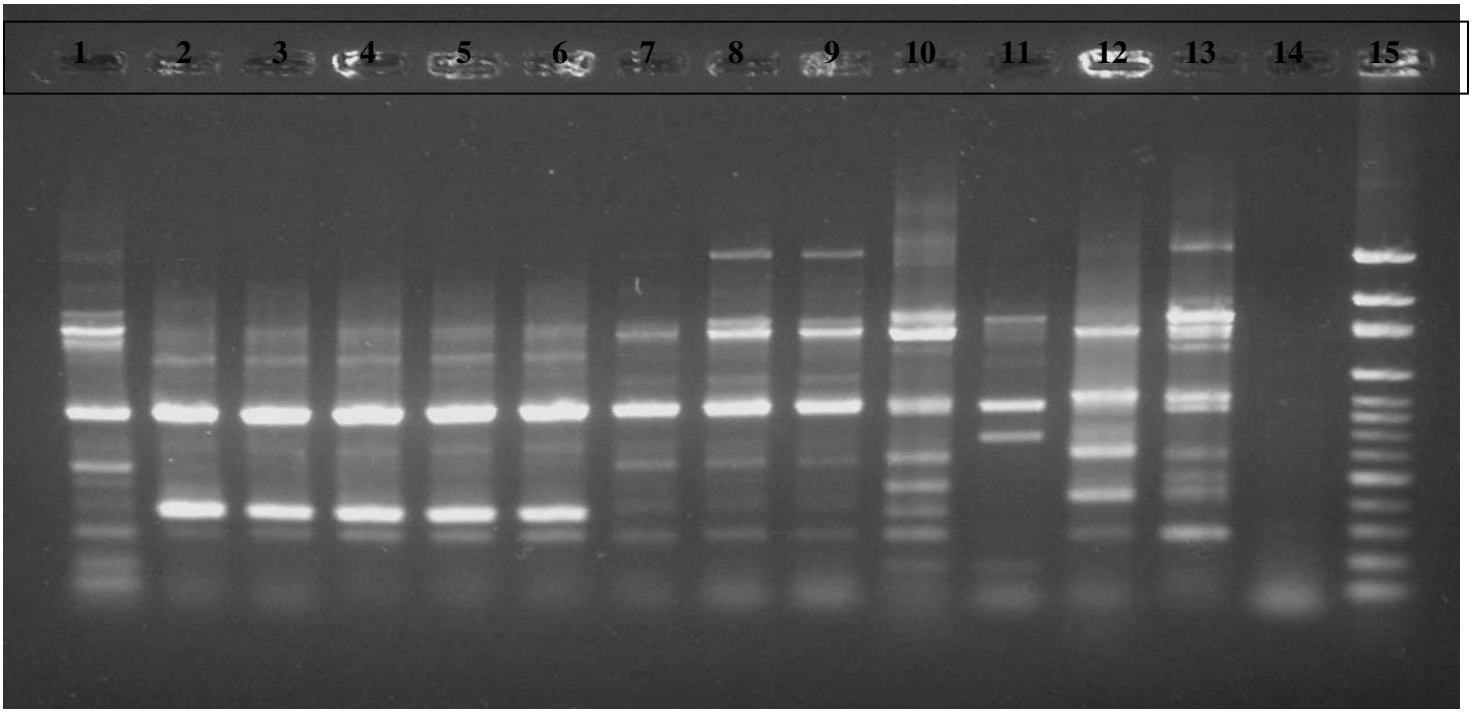
[B] Reference strain with the highest identity (range of % identities)

[C] Number of nucleotides compared

[D] Isolates from Australia

[E] Isolates from Israel

Figure 5.1. Example of RAPD analysis with primers desciribed by Fan *et al*. Lane 1 = HF-51; Lane 2 = K4158C; Lane 3 = K4110A; Lane 4 = K4110B; Lane 5 = K4110F; Lane 6 = K4181B; Lane 7 = K5029B; Lane 8 = K5037A; Lane 9 = K5039H; Lane 10 = 6/85; Lane 11 = ts-11; Lane 12 = R; Lane 13 = F; Lane 14 = negative control; and Lane 15 = molecular weight marker.



CHAPTER 6

DISCUSSION AND CONCLUSIONS

K5054 was isolated from an atypical MG outbreak in commercial turkeys. MG isolates from poultry that exhibit reduced pathogenicity, transmissibility and immunogenicity have been described as atypical or variant. Most of these reports have been in chickens, but there are also reports from turkeys (2, 5, 13, 18). Atypical serology, very mild or absent clinical signs and lesions, as well as difficulty in making an isolation by routine culture methods have been reported. In these instances the confirmation of suspicious cases is very difficult.

In the case of K5054, routine monitoring for MG according to the NPIP serological surveillance program did not detect the MG infection in the hen flock until late in the outbreak, although the ELISA test showed MG titers in banked sera.

The K5054 isolate was characterized as similar to the house finch strains by multiple analyses. Close examination of RAPD patterns using primer sets described by Fan *et al.* (3) and Geary *et al.* (4), revealed that the patterns of K5054 and house finch isolates are not completely identical but very similar. Previous studies have established that the finch MG strain was clearly different from commercial poultry strains, vaccines and lab strains (9).

Wild house finches nest and feed around buildings and farms, so that they may be in contact with domestic poultry. The house finch strain has been shown not to be host-specific to the finch although transmission may be slow. There is growing evidence that songbird species other than house finches are susceptible to MG infection and disease (6, 7, 12). MG from house finches can also be transmitted from affected finches to chickens by natural (contact) methods but transmit to domestic poultry quite slowly and with little pathogenic effect (14, 15).

From the bioassay in Chapter 2, it appeared that infection with the K5054 strain results in seroconversion with very mild clinical disease; confirmation of the infection by isolation of MG may be difficult. The infection appears to spread very slowly between turkeys. This was mirrored in the clinical picture of K5054 in the commercial turkeys.

This may be the first recorded incident of a naturally occurring infection of a house finch-like MG strain in commercial poultry.

Inoculation of turkeys with K5054 sinus exudate in Chapter 2 resulted in seroconversion with the appearance of very mild clinical signs 42 days after the challenge. This suggests that K5054 is mildly pathogenic in turkeys, although the inoculum dose in the bioassay may have been very low. PCR was unable to detect MG in the sinus exudates used to inoculate the turkeys. In Chapters 3 and 4, infection of chickens and turkeys with K5054 also did not result in significant clinical signs or lesions indicative of MG disease. It can be concluded that K5054 is a relatively avirulent MG strain for chickens and turkeys.

It has been established that virulence, invasiveness and immunogenicity of MG strains are directly correlated (11). Some live vaccines may be so mild as to be incapable of eliciting long lasting protective immunity.

K5054 was shown to elicit protective immunity in turkeys and chickens. Turkeys and chickens inoculated with K5054 were consistently protected against subsequent challenge with a virulent MG strain.

Also, despite the heavy challenge, R strain was re-isolated less frequently from birds that were vaccinated prior to challenge than those that were unvaccinated, although the difference was not always significant. Vaccination with K5054 may prevent subsequent infection with a virulent MG strain.

A vaccine strain should persist and multiply in the trachea long enough to elicit a protective immune response (16). The duration of immunity elicited may be associated with the colonization and persistence of the vaccine in the respiratory tract. K5054 was consistently re-isolated from the tracheas and less consistently from the air sacs of chickens and turkeys vaccinated with K5054 (Chapters 3 and 4). K5054 persisted in the upper respiratory tract and elicited protective immunity for the duration of the study (7 months) in Chapter 4. With respect to persistence K5054 seems to be comparable to F strain and ts-11.

K5054 did not spread among the turkeys in the bioassay (Chapter 2). K5054 also did not transmit to in contact chickens in Chapter 4 although it was re-isolated from all of the vaccinated birds (seeders).

This suggests that this strain was not as highly transmissible between turkeys or chickens as with MG in house finches. It has been suggested that the MG of finches

naturally has a low rate of transmission and that the rate of transmission is density-dependent (8) resulting in the seasonal fluctuation of the house finch MG outbreak (1).

The K5054 strain may be different from the MG isolates from house finches in its ability to spread to and infect domestic poultry. There may be future isolations of MG house finch-like strains from commercial poultry as the characteristics of house finch strain changes over time. On the other hand, this infection may have occurred under extreme or unusual circumstances, making it an incidental finding. However, there were no obvious confounding circumstances (e.g. moribund finches) observed at the farm.

It must be noted that K5054 did spread, albeit very slowly, among the commercial turkeys from which it was originally isolated. The K5054 infection in the turkeys challenged in Chapter 2 may never have reached the titers necessary for transmission to the contacts. A greater challenge to the seeders or a higher proportion of seeders in Chapter 4 may have allowed the transmission of K5054 to contacts. It can be concluded however, that K5054 has a relatively low rate of transmission.

In this study (Chapter 4), R strain was transmitted to many of the direct contacts (92%) although it did not spread to the indirect contacts. In our experience with R strain, in some instances it spreads very well to contacts and sometimes it does not. The conditions of this study may not have been ideal for the transmission of MG. The transmission of MG strains may be variable and unpredictable from study to study. Whithear et. al., was unable to show transmission of ts-11 to contacts (17) although in a different study Ley et. al., showed transmission of ts-11 to 0-40% of commingled pullets (10).

As described in Chapter 4, K5054 did not increase in virulence after ten passages in chickens and was also genetically stable; there was no change in the RAPD pattern or DNA sequence of selected genes.

We believe that a naturally occurring MG strain of low virulence, such as K5054, may have the advantage of increased stability over many *in vivo* passages as compared to laboratory attenuated strains. F strain is likely to have originated from a naturally occurring strain of moderate virulence and there is no published evidence of an increase in virulence although it has been used for many years. However, the possibility of "escape" and some increase in the level of virulence cannot be ignored with any live mycoplasma vaccine in poultry production situations. Diligence should always be applied when using a live vaccine; which can spread, multiply and change its characteristics when given the opportunity, however small the possibility.

To conclude this part of the studies, K5054 appears to be not only a safe and efficacious vaccine but also to be stable following ten *in vivo* passages, it has a low rate of transmission, the vaccine persists in the upper respiratory tract for long periods, and a single vaccination results in long lasting immunity. Further study is needed to evaluate the amenability of this strain to commercial production and application.

In the study described in chapter 5, DNA sequence analysis of selected genes was compared to RAPD analysis. The ultimate goal of researchers involved in MG strain differentiation and epidemiology is a method that will differentiate strains easily, rapidly and reproducibly from clinical samples.

It was found that sequence analysis any one sequence did not result in definitive identification of the isolates. However, when all three genes were analyzed the results were more useful and closely approached the discriminatory power of RAPD analysis.

The RAPD analysis and combined DNA sequence analysis data correlated well and there were no isolates that were classified as similar by the DNA sequence data that were identified as different by RAPD analysis.

There were some isolates that resulted in very similar RAPD patterns and differed very slightly in the nucleotide sequence (1 nucleotide change).

It appeared that the LP and *gapA* genes are more highly conserved than *pvpA* gene, resulting in fewer nucleotide changes in LP and *gapA* between isolates as compared to *pvpA*.

Some of the USA field isolates were very similar to vaccine strains (6/85, ts-11 and F strain) with respect to RAPD patterns and nucleotide sequences. The number of isolates that were similar to vaccine strains may not truly reflect the MG epidemiology picture in the US because vaccine-like isolates may be selected for further study thereby skewing the sample away from “wild-type” isolates.

The US isolates from house finches were closely related to each other and different from the reference strains and other field isolates with respect to RAPD patterns and DNA sequences. There was one US isolate from turkeys (K5054) that was also very similar to the house finch strains.

Other US isolates were not very similar to any of the reference strains; however similarities among isolates within and between MG outbreaks could be identified.

Many of the US field isolates that were not similar to a reference strain overall were very similar to a reference strain with respect to a single DNA sequence.

To conclude, RAPD analysis and combined DNA sequence analysis data correlated well, although comparison of any one DNA sequence did not result in definitive identification of the isolates.

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