AMERICAN UNIVERSITY OF BEIRUT

COMBINATION OF LIVE VACCINE AND PULMOTIL-AC IN CONTROL OF *MYCOPLASMA GALLISEPTICUM* IN BREEDER PULLETS

by
MAJD GHAZI KAIS

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Approved by:

Dr. Mohamad Talal Farran, Professor Agriculture	Advisor
Dr. Isam Bashour, Professor Agriculture	Member of Committee
Dr. Imad Saoud, Professor Biology	Member of Committee
Dr. Houssam Shaib, Lab Manager Agriculture	Member of Committee

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AN ABSTRACT OF THE THESIS OF

Majd Ghazi Kais for Master of Science
Major: Poultry Sciences

Title: Combination of live vaccine and Pulmotil-AC in control of *Mycoplasma gallisepticum* in breeder pullets.

This study aimed to determine the optimal time interval between the vaccination with a live *Mycoplasma gallisepticum* vaccine AviPro® MGF and the administration of the antibiotic Pulmotil® AC (PAC) to broiler breeder pullets while preserving the vaccine efficacy by studying the *Mycoplasma gallisepticum* F-strain (MGF) re-population pattern in the vaccinated birds after the PAC treatment was completed.

A total of 108 sixteen-weeks-old breeder pullets of the ROSS 308 strain were subdivided equally into 6 treatments of 18 birds. Pullets of treatment 1, the control, remained unvaccinated and deprived of Pulmotil® AC. Birds of treatments 3, 4, 5 and 6 were vaccinated with AviPro® MGF at 16 weeks of age via drinking water and administered Pulmotil-AC as per the manufacturer recommendation at 3, 7, 14, and 21 days post-vaccination. Treatment 2 was kept as the vaccinated PAC-deprived group. The pullets were tested by serology (ELISA) and quantitative PCR for the presence of MGF strain in the trachea at different days after the PAC treatment completion namely at 3, 7, 14, 21, 28 and 35 days post vaccination. All PAC-treated groups were showing tracheal MG recolonization after the antibiotic administration was discontinued, which indicates that the Mycoplasma strain of AviPro® MGF endures Pulmotil® AC treatment. The percentage of positive tracheal MG swab cultures was consistently higher in treatment 6, reaching a plateau at 14 days post PAC treatment discontinuation (100%, $P< 0.05$). These results were further reflected by the tracheal MG CFU counts which indicated better recolonization efficiency for birds of treatments 5 & 6 reaching up to 2322×10^3 and 2839×10^3 cfu/ml of broth, respectively at 35 days post PAC treatment discontinuation. ELISA test showed low titers indicating that the immunogenicity was not high enough to trigger abundant IgG production in all treatments. However, treatment 6 showed the significantly highest titer, recording a value of 2160, followed by the vaccinated PAC-deprived treatment 2 (1128). It is concluded that AviPro® MGF-vaccination of 16-week-old breeder pullets followed by PAC does not eliminate the F vaccine strain from the vaccinated treatments, and the earliest tracheal re-colonization of MGF was observed when the treatment with PAC started at 21 days post vaccination.

Keywords: *Mycoplasma gallisepticum*, AviPro MGF, Pulmotil AC, pullets, qPCR, ELISA

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ABBREVIATIONS

%	Percent of a Hundred
AREC	Agriculture Research and Education Center
AUB	American University of Beirut
CEF	Chicken Embryo Fibroblast
Cfu	Colony forming unit
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
eg.	Example
ELISA	Enzyme-linked Immunosorbent Assay
EP	Egg Production
FCR	Feed Conversion Rate
Fig.	Figure
HI	Haemagglutination Inhibition
IgG	Immunoglobulin G
M	Mycoplasma
MG	*Mycoplasma gellisepticum*
MGF	*Mycoplasma gellisepticum* F-strain
MRL	Maximum Residue Level
MS	*Mycoplasma synoviae*
n.d	Not determined
NDV	New Castle Disease Virus
IBD	Infectious Bursal Disease
IBV	Infectious Bronchitis Virus
mg	Milligram(s)
ml	Milliliter(s)
g	Gram(s)

µg	Microgram(s)
L	Liter(s)
cm	Centimeter(s)
xg	Times gravity
bp	Base pair
®	Registered trade mark
qPCR	Quantitative PCR
et al.	*et alia* (and others)
°C	Degree Celsius
pH	Hydrogen Potential
µm	Micrometer(s)
USA	United States of America
N.Y.	New York
Ltd.	Limited
w/v	Weight to volume
psi	Per square inch
N	Normal Concentration
NaOH	Sodium Hydroxide
I.U.	International Unit
dpp	Days post pulmotil treatment
dpv	Days post vaccination
Inc.	Incorporated
RT-PCR	Real-time PCR
TMB	3,3’,5,5’-Tetramethylbenzidine
nm	Nanometer(s)
S/P	Sample to positive ratio
PCx	Positive control mean
NCx	Negative control mean

Mgc2	Adhesion coding protein of MG
IACUC	International Animal Care and Use Committee
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
trt.	Treatment
spp.	Several species
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
i.e	That is
CRD	Chronic Respiratory Disease
IgA	Immunoglobulin A
RAPD	Random Amplified Polymorphic DNA
Dis.	Disease
Tech.	Technology
Vet.	Veterinary
Med.	Medicine
rpm	Rounds per minute
Sci.	Science
NPIP	National Poultry Improvement Plan
PAC	Pulmotil-AC
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
SPA	Slide Plate Agglutination

To My

Beloved Parents

CHAPTER I

INTRODUCTION

Mycoplasma gallisepticum (MG) is a wall-less prokaryote that can infect both human and animals leading to chronic respiratory diseases in poultry chickens and sinusitis in turkeys (Winner, Rosengarten and Citti, 2000). In addition, the economic losses incurred by MG infection are due to decreased egg production and size, decreased feed efficiency, mortality and carcass condemnations. MG can be transmitted vertically from an infected parent stock to its progeny through eggs and horizontally through human activity, feed, water and airborne. This disease is established when the microbe attaches to the host cell by a process called adhesion. Post attachment, MG infection discharges mucous granules, followed by the exfoliation and annihilation of both ciliated and non-ciliated epithelial cells contributing to colonization of the respiratory tract.

The prevention of MG infection in poultry necessitates the application of strict biosecurity measures and the use of either live or killed vaccine to boost the immune response against this pathogen. The use of killed vaccines might not confer protective antibody level to the flock as the produced IgG immunoglobulins cannot easily cross the mucosal barrier at the surface of which MG resides. Live vaccines showed significant impact in protecting breeders and layer flocks such as the mutant ts-11 and were more promising than the killed ones, yet the level of protection conferred by these vaccines to poultry flocks is not consistent (Jacob *et al*., 2014; Ferguson-Noel *et al*., 2012).

This study combines the use of antibiotics, namely tilmicosin (Pulmotil AC),

and a live MG vaccine of the F strain in order to develop a sound protocol for the control of MG in breeder pullets. This study aimed to determine the optimal time interval between the vaccination with a live MG vaccine AviPro® MGF and the administration of the antibiotic Pulmotil® AC (PAC) to broiler breeder pullets while preserving the vaccine efficacy.

CHAPTER II

LITERATURE REVIEW

A. Overview: Mycoplasma gallisepticum in Poultry

There are several forms of avian mycoplasmosis that affect poultry. One of those is *Mycoplasma synoviae* (MS) which occurs less frequently than MG. *Mycoplasma synoviae* causes an infection known as infectious synovitis. It is considered to be an infectious disease common to turkeys and chickens, like MG; however, MS is characterized by an infection of the "synovial membranes of joints and tendons sheaths" (Khalifa *et al*., 2013).

MS and MG have been associated with severe airsacculitis in poultry. Both infectious diseases affect the hatchability and quality of production, the growth rate, the reproduction rate and the health of chickens and turkeys (Khalifa *et al*., 2013).

Across the years and due to an alarming amount of economic disrepair, these avian mycoplasmosis were diagnosed through serological assays. These serological tests included "the rapid slide agglutination test, the haemagglutination inhibition test, and ELISA. Culture techniques are laborious, time consuming, expensive and require sterile conditions. Problems experienced with culture include overgrowth by faster-growing bacteria, or failure in subculture growth. Particularly in difficult cases, *in vivo* bioassays are necessary and involve the inoculation of specific pathogen-free chickens with suspect material" (Khalifa *et al*., 2013).

Mycoplasma gallisepticum is the most pathogenic of all avian mycoplasma. It lacks cell wall and resists immunological responses. The bacterium works by having membrane surface proteins, also known as adhesins that attach onto the receptors of the

host cells where they take over the cell function via their infection. In addition, these adhesions act as virulent factors which play a role in antigenic variation and immune evasion (Ley, 2018).

Mycoplasma gallispeticum infections are common amongst chickens and turkeys mostly with several cases of infections targeting pheasants, peafowl, pigeons, quail, ducks, geese and wild house finches (Ley, 2018).

This short overview merely creates an understanding of the disease and why it is so prevalent to study. According to Levisohn and Kleven (2000), in the earliest contemporary study about the disease, "Infection with MG has a wide variety of clinical manifestations, but even in the absence of overt clinical signs, the economic impact may be significant. The most dramatic disease presentation of MG is chronic respiratory disease in meat-type birds, often as one of several aetiological agents in a multi-factorial disease complex. Transmission of MG in ovo from infected breeder birds to progeny is the major route of dissemination of the infection, and is the prime consideration for international trade." This is what makes it imperative to note its patterns and find appropriate, low-cost, en masse treatments.

B. Pathogenesis & Intracellular Interaction

Mycoplasma gallisepticum is a species belonging to the bacteria kingdom. They are slightly gram-negative, ovoid cells that lack cell walls are merely bound by a plasma membrane. They are anaerobic cells and rely on sterols for growth (Papazisi *et al.*, 2003). The bacterial cells possess minimal genetic material and are also miniscule.

The fact that a cell wall is not present and that the plasma membrane protects minimal genetic material accentuates the interdependence found between the bacteria and the host animal's cells. Additionally, the speed with which the chronic infection

occurs could be attributed to the "phenotypic variation of major surface antigens occurring at high frequencies" (Levisohn and Kleven, 2000).

These prokaryotes enjoy being hosted by their immune-competent host because it provides them unlimited resources for growth, a defensive protection against their own hosts' immune system and a continued existence. These types of avian diseases were left unknown for quite some time with the most intricate study being developed early in the 21st century. This proves that these prokaryotes have enjoyed a strong sense of evolution and can continue to be crucial in their operation (Winner *et al*., 2000).

C. Strains and Virulence

1. Virulent Strains

To understand the biological method of their operation, a study was carried out by Winner *et al*. in 2000. This study reflected the robustness of MG cells; it also showed their intracellular attitude once the invasion takes place. Below is a summary of the study's results:

"One approach that may enable a better understanding of the interaction occurring between MG and its host cell is the use of cultured monolayers of established cell lines, which offers a less complex environment than that of the actual target tissue. In the present study, we used human epithelial cells and chicken embryo fibroblasts (CEF) as a model system to demonstrate that *MG* strain R may indeed act as a facultative intracellular microorganism, with the virulent low-passage population R-*low* and the avirulent high-passage population R-*high* showing differences in their invasion frequency that increase after multiple passages through cultured cells."

The virulence of MG differs from one strain to another, also it depends on the medium in which these prokaryotes are propagated in when infection happen in vitro.

There are two prototypes of R strain, the low passage population R-low which causes lesions in the air sac, and the high passage population R-high which causes tracheal colonization (Winner *et al*., 2000).

The time needed to invade a eukaryotic cell by R-low was found to be between five minutes to forty-eight hours. On the other hand the persistence of MG inside the cell remained for 48 hours, till the death of the infected cell (Winner *et al*., 2000).

2. Mild and Non-Virulent Strains

F-strain is more pathogenic to turkeys than chickens. The ts-11 and the 6/85 strains are less pathogenic to turkeys and chickens than the F-strain while house finch strains of MG have shown relatively low pathogenicity for chicken and turkey (Ley, 2018).

D. Epidemiology

The most frequently infected poultry are chickens and turkeys. There are two main channels of infection (Levisohn and Kleven, 2000):

1. Vertically

This insinuates that the transmission occurs from the breeding flock to their offspring (in ovo).

2. Horizontally

This kind of transmission occurs from infected birds to uninfected birds either through direct contact or through contact with the waste of those infected bird. Additionally, horizontal infection can occur “via infectious aerosols and through

contamination of feed, water, and the environment, and by human activity" (Ley, 2018).

The specificity of the host bird and the frequency of the asymptomatic infection in the environment of the uninfected birds play an important role in the epidemiology of the disease.

Additional factors that increase the risk of transmission are:

- Cold weather
- Poor air quality
- Crowded spaces
- Live vaccination

As mentioned earlier, the mode of detection of the disease took a long time to reach sophistication and high accuracy levels. Commonly, infection would be detected through clinical methods. According to Ley's research in 2018, the detailed process of clinical detection goes as such: "Serology by agglutination and ELISA methods are commonly used for surveillance. Hemagglutination-inhibition is used as a confirmatory test, because nonspecific false agglutination reactions may occur, especially after injection of inactivated oil-emulsion vaccines or infection with MS. *Mycoplasma. gallisepticum* should be confirmed by isolation from swab samples of infraorbital sinuses, nasal turbinates, choanal cleft, trachea, air sacs, lungs, or conjunctiva. Primary isolation is made in mycoplasma medium containing 10%–15% serum (Figure 1). Colonies on agar medium are used for species identification by immunofluorescence with species-specific antibodies. PCR can also be used for detection of MG DNA using swabs taken directly from infected sites (choana, sinuses, trachea and air sacs) or after growth in culture" (Ley, 2018).

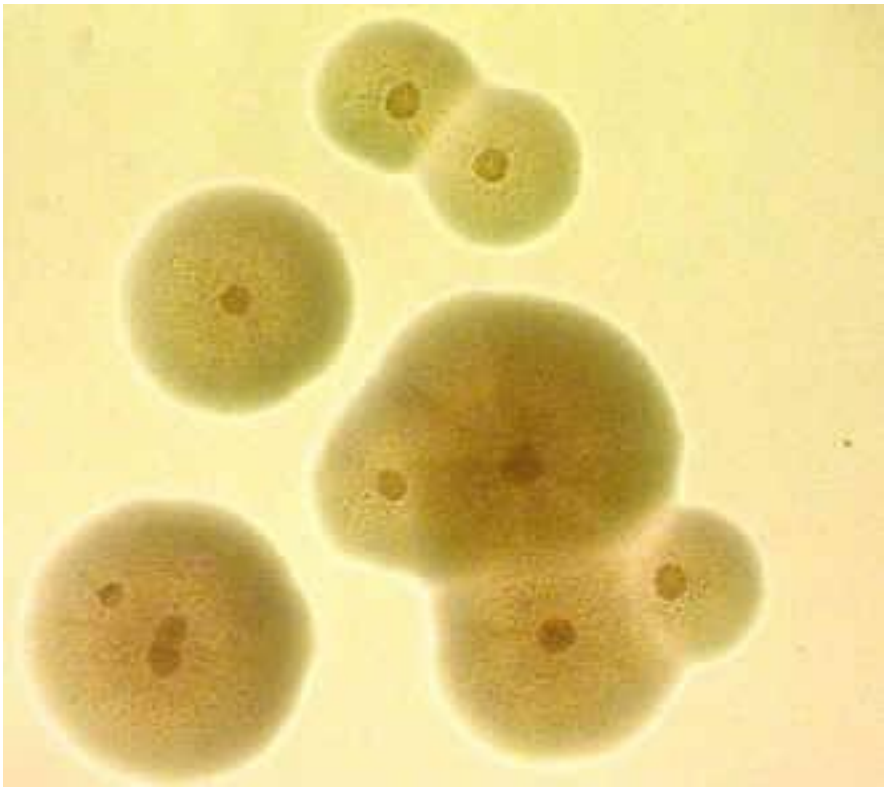

Fig. 1. *Mycoplasma gallisepticum*
Source: Papazisi, L., Gorton, T.S., Kutish, G., Markham, P.F., Browning, G.F., Nguyen, D.K., Swartzell, S., Madan, A., Mahairas, G. and Geary, S.J. (2003). *Microbiology 149*(9):2307-16.

In addition to cultural methods, detection includes serology and DNA based techniques:

3. Serology

Many factors can affect the accuracy of a serology test like the timing of the test, the size of the flock, and the time interval between the testing.

a. Haemagglutination Inhibition (HI)

Haemagglutination happens when the blood cell receptors bind to an antigen resulting in clumping. This test is highly specific to the level of strain differentiation.

However, the major disadvantage of this test is that it cannot differentiate between infectious and noninfectious antigens because both are able to cause clumping.

b. Slide Plate Agglutination (SPA)

This test can be performed in the absence of clinical MG signs because it can detect the immunoglobulin M that is produced upon infection. This kind of tests is usually inexpensive and highly sensitive but with low specificity it can encounter false positive results. Sometimes other confirmatory serological tests are required due to the presence of cross reactions.

4. DNA based Methods

Polymerase chain reaction represents rapid and sensitive results upon testing for a specific pathogen. The major advantage of PCR technology is that it can be used to diagnose wide range of pathogens that infect poultry species. The results are usually obtained within one day. The accuracy of this test is reflected even if the host is co-infected or contaminated with other diseases. Pooling the samples when using the PCR techniques decreases the testing's cost. PCR technology is recommended to check for the presence of *M. gallisepticum* because its implementation can detect MG in embryonated eggs, due to its high sensitivity. Moreover, the availability of commercial *M. gallisepticum* PCR kits is able to differentiate between the F-strain and other strains of MG (Levisohn and Kleven, 2000). Moreover, there are various advantages to the use of Random Amplification of Polymorphic DNA-PCR (RAPD-PCR) for identifying strains and isolates: 1) extensive knowledge of the biochemistry or molecular biology of the species under study is not required; 2) RAPD-PCR does not require functional selection based on a specific target gene; 3) the method allows the examination of large

number of samples at once and in a short time (Marois *et al*., 2001).

Mycoplasma isolates need to be diagnosed by species not as a spectrum of their symptoms because certain birds can be infected by nonpathogenic mycoplasmas. Also, "*E coli* infection, Newcastle disease, avian influenza, and other respiratory diseases (eg, infectious bronchitis in chickens) should be considered in the differential diagnosis and can act as inciting or contributing pathogens" (Ley, 2018).

E. Symptoms and Effects

Initially, MG affects the epithelium of the conjunctiva, the nasal passages, sinuses and trachea in its conquest across the poultry's cellular composition. As the disease spreads further, the acuteness and severity of the infection reaches the bronchi, air sacs and eventually, the lungs of the infected animal (Ley, 2018). This severity of infection is what distinguishes MG from similar respiratory infections such as *Escherichia coli* and MS.

There are uncomplicated versions of MG. This milder form can affect the catarrhal sinusitis, tacheitis and airsacculitis (Figure 2). The more severe the infection, the more there are risks for exudative accumulations, adhesive pericarditis and fibrinous perihepatitis. The mucus membranes thicken and become invaded with inflammatory cells. These symptoms can lead to further complications with lymphoid hypoplasia and germinal center formations (Ley, 2018).

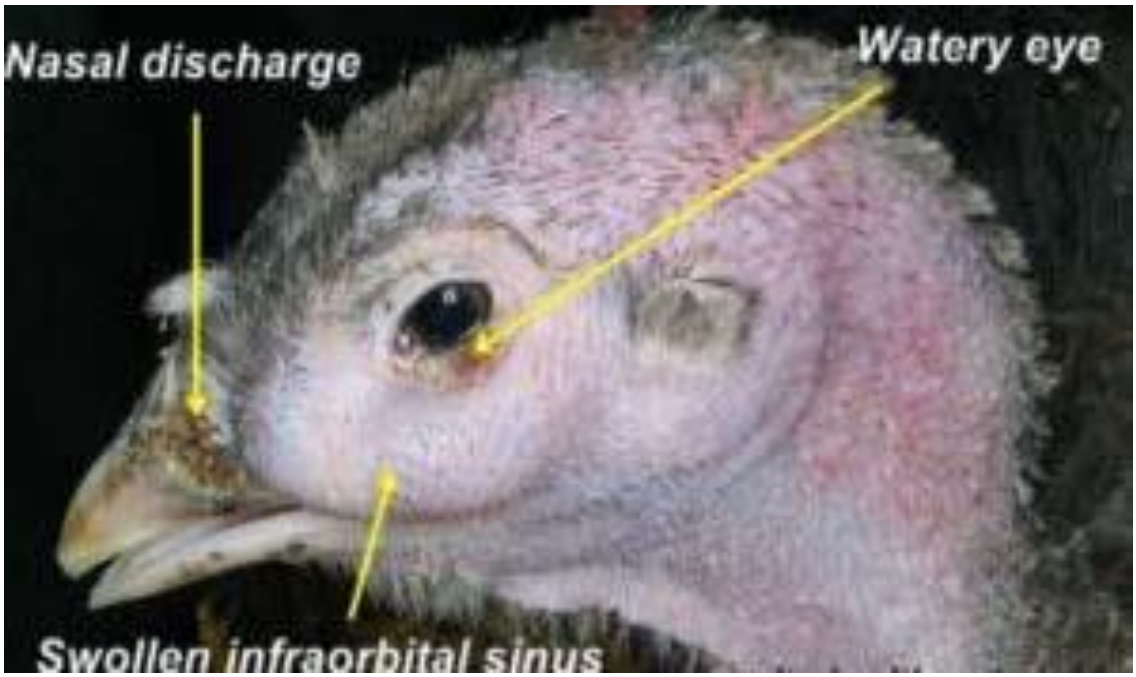

Fig. 2. Milder Effects
Source: Parsons, D. (2017). Mycoplasma gallisepticum; available from https:// poultryhealthcentre.com/mycoplasma-gallisepticum; Internet; accessed 20 August, 2018.

Additional effects & symptoms involve yellow pus in sinuses, swollen heads due to excessive lacrimation, froth in the eye, ataxia (mostly in turkeys), infection of the oviduct in laying hens, tenosynovitis & arthritis, poor productivity, ocular discharge, abnormal feather, leg problems, reduced chick viability (mostly in chickens), coughing and depression (mostly in house finches) (Parsons, 2017).

The most unsettling result of infection is that, birds that are infected with MG will continue to be carriers of the disease for the rest of their lives (Moyle *et al*., 2015). However, vaccinations and antibiotics have made the field safer and less infectious for chickens and turkeys. These vaccines are essential because the rate at which the disease travels is fast.

F. Common Statistics & Facts

- Chickens vaccinated with an F-strain MG vaccine (discussed later in the literature review) can be re-vaccinated with no adverse effects (Leigh et al., 2010).
- Post-infection, the average rate of transmission from one infected chicken to the other ranges from one to few weeks.
- *Mycoplasma gallisepticum* does not affect humans who eat the meat or eggs of infected birds, however birds that have been treated with antibiotics should not have their eggs consumed before 10 days post treatment discontinuation (Moyle *et al*., 2015).
- *Mycoplasma gallisepticum* can lead to a reduction of 6.3% in egg production (Peebles & Branton, 2012).

G. Prevention and Control

The primary control program of MG starts by having a breeding stock free of mycoplasmosis, in addition to the concepts set by the national poultry improvement plan (NPIP): routine serological confirmatory tests and the direct eradication of the infected breeding bird to avoid egg transmission.

Upon infection, suitable medication treatments rise as an option for the control of the disease. Antibiotics can help in decreasing the rate of egg transmission and improving the clinical signs.

An effective control method of mycoplasma is to have continuous monitoring or screening system. This will help in early detection of the disease thus prevention of its spread to other birds in the flock. Moreover, upon rearing breeders the selected males should be mycoplasma free. The NPIP suggests monitoring every 60-90 days, and to perform serological testing before the onset of egg production (Kleven, 2019).

1. Killed Vaccines

Bacterins are killed or inactivated vaccines. The administration of MG bacterins to chicken is somehow debatable due to the presence of contradicting results according to studies previously done. Hyman et al (1989) found that the use of this killed vaccine can help in reducing egg transmission and egg production losses in addition to its prevention to lesions and respiratory signs. On the other hand, Kleven (2019) found that bacterins had no effect on the population of MG in the respiratory tract. Bacterins are considered to be safe due to the fact that they are killed; they require more than one dose to be effective. The major disadvantages are the inability to colonize and displace the wild strain in the trachea (Kleven, 2019). Bacterins should be given to each bird making it impractical and expensive in terms of labor and cost, add to this there are some reports indicating the presence of infection at the injection site (Kleven, 2019).

2. Live Vaccines

There are three different strains of MG used as live vaccine: F strain, 6/85, and ts-11.

a. <u>F Strain</u>

The F strain was isolated by Yamamoto and Adler in 1958 and latterly used as a vaccine. MGF is known for its low to moderate virulence on chicken breeders and layers, but of high virulence to turkeys and broilers (Lin *et al.*, 1982). This strain can persist in the upper respiratory tract, and is capable of decreasing egg production losses in layers. Moreover, it has the ability to displace the virulent R-strain of MG thus making the chicken resistant to it (Levisohn and Dykstra, 1987). Usually this vaccine is

administered through several routes eye drops, drinking water, spraying or intranasal. However, the timing of vaccination is at 8-14 weeks of age, before the onset of egg production, because F strain can be transmitted through eggs (Kleven, 2019).

The F strain of MG is an effective vaccine. However, vaccinations need certain conditions to be deemed successful. Vaccination success depends on the age of the bird at the time of vaccination, particularly when the layers are administered vaccines at the prelay period (Jacob *et al*., 2014).

A single F strain of MG vaccine during the prelay period has been the norm in the poultry industry. "It has been demonstrated that the inoculation of pullets with FMG may lead to a subsequent delay in the onset of lay and a decrease in post-peak egg production (EP) when compared with un-inoculated MG clean hens. Conversely, under commercial conditions, an increase in the EP of FMG-vaccinated layers was reported when compared with unvaccinated birds that were infected with a field-strain of MG (Jacob *et al*., 2014).

Overlay vaccines must be given later throughout the life of the birds, during the post-peak lay period to achieve and maintain a long-lasting protection. As mentioned earlier in this literature review, the birds must be vaccinated constantly, not just once, due to the nature of the bacteria's spread and its continuous evolution.

The administration of FMG during post-peak lay leads to adverse effects on the physiology of the birds. They do not show up in the case of administering the vaccine at early stages of the birds live namely at 8-14 weeks of age or as early as 6 weeks of age. In parallel, the occurrence of MG in later stages of life is more detrimental to the bird than if it has been contracted at a younger age (Jacob *et al*., 2014).

Based on the studies of Liu *et al.* (2013) conducted on birds that have been vaccinated by the F strain of MG, we can generalize the following pattern of effects

noted:

- Egg production and breeding speed in vaccinated hens is faster than hens that aren't vaccinated.
- Eggs from vaccinated hens have a greater eggshell thickness and less incidence of blood-meat spots.
- Eggs from vaccinated hens have better hatchability than the non-vaccinated birds.
- Air sac lesions of birds that are vaccinated are dramatically less than in unvaccinated birds.
- Uterus of vaccinated hens are usually longer and healthier than those of vaccinated birds
- Overall productive and reproductive function is improved by administering vaccinations.
- Very few genomic differences occur due to vaccination but there is a noted mutation in cytadherence-related proteins in escaped mutants (Szczepanek *et al*., 2010).

b. 6/85 Strain

The 6/85 strain has some similar characteristics to the F strain such as the ability to displace wild strains, the capability to decrease egg production losses, and it can be found in the upper respiratory tract, but without inducing any immune response. It was discovered in 1992 in USA (Kleven, 2019). It can't be transmitted easily from a bird to another. This vaccine is sold in more than ten countries and administered by spraying the commercial layer chickens.

c. ts-11Strain

ts-11 is a chemical mutant of MG; it was described by Whithear *et al*. (2000) in Australia, and currently sold in more than 20 countries including the US. Similar to the above mentioned live vaccines, ts-11 colonizes the upper respiratory tract -because it is sensitive to temperature grows best at 33°C- inducing a long lasting immunity to the bird. This avirulent strain is administered intraocular, and has low ability to spread among birds.

d. Recombinant Fowl Pox Vaccine

Recombinant MG vaccine was described by Biomune. There are no published data about its safety and efficacy. It expresses MG proteins thus not considered a live vaccine (Kleven, 2019).

Levisohn and Kleven (2000) reported that when commercial layers were infected with the R-strain of MG, only the F strain vaccine that was administered to the chickens had the ability to displace the virulent wild strain but not the F-strain, unlike the two other types of live vaccines, ts-11 and 6/85. As a result ts-11 vaccine strain was administered only once to the new commercial flock as a trial to eradicate the F-strain. Serological test revealed that those layers were found to be MG negative.

According to unpublished data collected from field experience (Kleven *et al*., 2019), 6/85 strain has the ability to displace the F strain and produce MG free flocks over years. As a conclusion F strain should be used to get rid of the field strain, and after that the milder strain should be employed for the production of MG free flocks.

H. Antibiotics and Treatments of *Mycoplasma gallisepticum*

The poultry industry, broilers or layers, is growing rapidly. This increase

necessitates the use of feed additives to cope with their growth performance, as well as prevention and treatment of diseases. These additives are now considered a part of the food-pyramid. However, their excess in poultry feed can accumulate in the processed poultry products which is usually controlled and regulated by law (Mund *et al*., 2017).

Feed additives like antimicrobial agents started to be used in the mid of 1950s as growth promoters and inducers of egg production. Some examples of antimicrobial agents that were used back then include: penicillin, chloramphenicol, tetracycline, virginiamycin, tylosin and avoparcin (Mund *et al*., 2017). The mechanism of action of these antimicrobial agents in not well known, but it is suggested that they have activities against harmful bacteria and pathogens like those that are present in the intestines leading to a better absorption of nutrients.

Nowadays the use of antibiotics as growth promoters is banned in developed and some other developing countries. Residues are formed and accumulated in the liver, muscles and kidneys when they are not metabolized or absorbed by the animal (Zhang *et al*., 2004). The overdose of these additives may cause drug toxicity, develop antibiotic resistance, and become carcinogenic and teratogenic.

When the drug is administered to the bird, it circulates in the blood and reaches the ovaries, the place where the egg formation takes place, hence the deposition of the antibiotic to the albumin and the yolk. This was reported by Amiri *et al*. (2014) and Al-Ghamdi *et al*. (2000) when they found residues of nitrofuran, used for salmonellosis treatment in eggs. Likewise, deposits of sulfonamides, tylosin, oxytetracycline, streptomycin, sulfaquinoxaline and many other drugs were reported in egg samples. The contamination of poultry meat is also reported and demonstrated by several researchers in which they found residues of quinolones, enorflaxin, macrolides, beta-lactams and others in poultry meat.

The efficacy of antibiotics used to treat mycoplasma diseases is decreasing due to antibiotic resistance. Zanella *et al*. (1998) reported the effects of various antibiotics (erythromycin, spiramycin, streptomycin, tylosin, enrofloxacin and tetracyclines) used in the treatment of avian mycoplasmosis. They found that the resistance of MG is due to the nature of the antibiotic used. Moreover, the tolerance to streptomycin, erythromycin, spiramycin and tylosin was found after 3, 5, 6 and 9 passages, respectively.

There are several antibiotics that have been known to reduce the symptoms of MG. These antibiotics however, have shown mitigation in the severity of the symptoms yet they were not successful in eliminating the disease. Such antibiotics are (Moyle *et al.*, 2015):

- Tylosin
- Fluoroquinolones
- Tiamulin

Another layer of complication to the utility of certain antibiotics is their effect on animals that are raised for meat and eggs, as these antibiotics can alter the quality of poultry products and affect the birds' performance.

Even if birds are treated with antibiotics, they can still show illness signs and can further spread the disease. A negative economic effect of antibiotic treatment is the continuous need to keep administering the medication. It is not a one-time treatment.

Additionally, farmers and keepers of flocks must ensure that all equipment is rigorously cleaned or completely changed and that the entire environment is disease-free. Most consultants suggest that instead of treatments and continuous medication, farmers should merely clean and disinfect the entire area and start with a brand new flock, saving time and costs.

This suggestion of starting with a brand new flock stems from the several pessimistic facts that the disease persists on the chickens and other birds. The disease can still be contagious even when being treated, the farmland is too large to be completely cleared out from a micro-sized bacterium, and the disease remains present for a year or so after treatment (Dhondt *et al*., 2014).

An important study conducted in the Islamic Azad University in Tehran attempted to study and document the comparative results of utilizing two different antibiotics on flocks diagnosed with MG. The two administered antibiotics were Tiamulin and Tylosin. Below is an extract from the study that describes the sample group, methodology and materials used:

"In this study, 240 Ross 308 broilers divided in 3 groups, and each group is divided into 4 replicates with 20 birds each. In group-1 Tiamulin and group-2 Tylosin was used first in days 3, 4 and 5 then later in days 19, 20 and 21. The dosage of Tiamulin and Tylosin was 100 grams in 200 Litters of water. In the last group, placebo was used and that group was labeled as a control group. This study was performed in 42-day period and gross lesions, mortality, and growth parameters including body weight gain, feed intake and feed conversion rate (FCR) were calculated in all groups weekly" (Feizi *et al*., 2013).

The results of the study showed that antibiotic use can reduce the severity of the disease and protect against large economic losses mainly due to the fact that the only thing in common amongst both administered antibiotics was the improvement in broilers' performance and life span (Feizi *et al*., 2013).

For example, antibiotics including Macrolides, Lincosamides and other tetracyclines inhibit protein synthesis. They are all used for the treatment of MG. Nevertheless, all these above mentioned antibiotics only showed an improvement in the

symptoms but no eventual elimination of the disease. These treatments reduce the economic losses because of the decrease in carcass condemnations.

The study conducted by Feizi *et al.* (2013) highlighted several different antibiotic usages across the world and how each one of these was effective in its own way and in regard to the flock that was being treated. It is safe to say that it is not the antibiotic alone that proves potent or not but also the genetic diversity, quality and type of flock that is being treated that affects the effectiveness of the treatment.

For example, Tylosin had the best effect on the respiratory tract symptoms that sprout due to MG. Lincomycin, Oxytetracycline and Spectinomycin alleviated the disease symptoms but not in the same way as Tylosin, probably because of its mode of action. Of all the listed antibiotics, the study found that "chicks that were infected with Mycoplasma and treated with Tiamulin and Tilmicosin in comparison to control group had lower clinical signs, mortality and lesions in air sacs, and re-isolation rate of MG in treated groups were lower than control group, and body weight was significantly improved in treated groups" (Feizi *et al.*, 2013).

One of the most important and relieving effects of using some of the discussed antibiotics was the decrease in nasal discharge. A study by Arzey and Arzey in 1992 found out that remission of nasal discharge was achieved in "60% of hens treated with 100 mg oxytetracycline, in 100% of hens treated with 100 mg or 200 mg spiramycin, in 92% and 85% of hens treated with 100 mg tylosin, parenterally and orally, and in 89% and 88% of birds given 100 mg tiamulin and tylosin-dihydrostreptomycin, respectively."

The results have often shown improvement of the ailing symptoms, a complete remission of nasal discharge, better quality of carcasses due to improved symptoms and a general improvement. However, none of the antibiotics which come at a high price

with a long term plan of reapplication have totally removed the disease from infected flocks.

I. Pulmotil AC (Tilmicosin)

Pulmotil AC is a liquid antibiotic administered to birds infected with MG. The active substance found in Pulmotil AC is Tilmicosin as a phosphate, thus the reference to it as simply Tilmicosin (Figure 3). The excipients found in the drug are propyl gallate and disodium edetate amongst others.

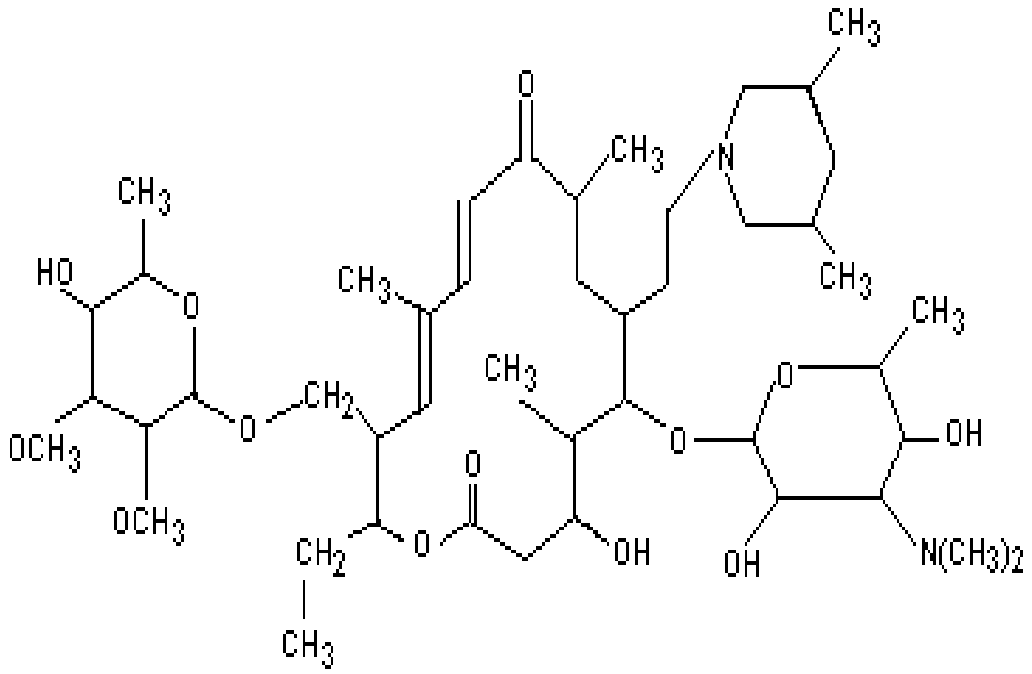

Fig. 3. Tilmicosin Chemical Structure
Source: Roberts, G. (n.d.). "Tilmicosin. Commonwealth Department of Health and Family Services"; available from http://www.inchem.org/documents/jecfa/jecmono / v38je06.htm; Internet; accessed on January 14, 2019.

Tilmicosin is a semi-synthetic antibiotic. It belongs to the macrolide group. It exerts its activity against MG by affecting the bacterial protein synthesis. At normal

dosage concentration, Tilmicosin has bacteriostatic action which becomes bactericidal at high concentration levels.

Tilmicosin works primarily against Gram-positive micro-organisms and MG that lacks cell wall which makes it easier for the chemical to diffuse inside the cell, through the plasma membrane and hinders the protein synthesis process. Mostly, the antibiotic functions against Mycoplasma of bovine, porcine, ovine and avian natures, especially MG and MS discussed earlier in this literature review.

The antibiotic comes in the form of a concentrated oral solution to be mixed with drinking water. It has a clear yellow/amber hue to it. The antibiotic can be specifically administered to chickens, turkeys, pigs and calves. However, as with other antibiotics, Tilmicosin must not be given to chickens that produce eggs for human consumption (Elanco Animal Health, 2009).

Tilmicosin must be administered as such:

To be included in the drinking water at a daily dose of 15-20 mg/kg bodyweight in chickens and 10-27 mg/kg bodyweight in turkeys for 3 days, which may be achieved by the inclusion of 75 mg tilmicosin per liter (30 ml Pulmotil AC per 100 liters).

The strength of Tilmicosin lies in its belonging to the macrolide group because scientific evidence informs us that macrolides act synergistically with host cells, enhancing phagocyte killing of bacteria. In addition, Tilmicosin is powerful because it has shown that it inhibits in vitro the multiplication of the porcine reproductive and respiratory syndrome virus in macrophages, in sync, killing potential and current MG (Elanco Animal Health, 2009).

According to the European Union and the Chinese Ministry of Agriculture, the maximum residue level (MRL) of tilmicosin in muscles, liver and kidneys of broilers

should be 0.075, 1.0 and 0.25 μg/g, respectively. The recommended withdrawal time should be ten days (Zhang *et al*., 2004).

J. Future Development

Of all the antibiotics discussed, Tilmicosin seems to be the most prevalent. However, other factors in the industry can affect the efficiency of the products being used to treat diseases such as MG. Many studies are currently being carried out to understand the effects of toxic symptoms that could arise from the administration of Tilmicosin to contaminated animals. Additionally, the Food and Drug Administration requires new tests to understand the bio-sustainability of utilizing this antibiotic. Regulations need to be set up on grander scales to appreciate and evaluate the use of drugs on cattle animals due to their transmission to human life, with a particular focus on the quality of the eggs that chickens produce (Abu-Basha *et al*., 2007).

A recent study conducted by Farran *et al*. in 2018 has brought more significant attention to the future development of the field. Even though the antibiotics used to treat MG do not seem to be able to completely remove the disease from the host body, this new combination of antibiotics proposed in this study seems to have alleviated the symptoms to near extinction and stopped the vertical transmission of MG from parents to progeny.

The study involved a 44-week trial of administering dosages of Tilmicosin and another antibiotic called Denagard. The results showed that the antibiotics cleared the MG tracheal colonization, reduced the vertical transmission rate to the offspring, improved fertility, increased egg weight and increased hatchability percentages.

The mixture of Tilmicosin and Denagard also proved a positive reflection on the chickens' immune system, relieved the flock's respiratory tissues "which reduced

the antibody titer to MG and left space for the immune system to increase its response against IBV, IBD and NDV vaccines" (Farran *et al*., 2018).

CHAPTER III

MATERIALS AND METHODS

A. Preparation of Poultry House

Walls, floors, and ceilings of an environmentally controlled poultry house at AREC were cleaned, flushed with water and disinfected by thoroughly spraying overnight with sodium hypochlorite. A prepared 10% sodium hypochlorite was placed in a platter (shoe-dip) at the entrance and at all points being used to access the poultry house to immerse and disinfect shoes prior to placing on a shoe cover and entering the room; and a new batch was added when needed. Gloves, lab coats, head covers, masks and biosecurity measures were strictly used. The house was separated into 6 pens. Wood shavings were spread on the floor of each pen at a depth of 5 cm. Each pen was supplied with one feeder and one drinker. Feed was offered once a day as per breeder manual recommendations, and water was offered *ad libitum*.

B. Birds and Treatment

A total of 108 ten-week-old pullets of Ross 308 strain were divided into 6 treatments of 18 pullets each. Birds of treatment one, the control, were not vaccinated nor treated with Pulmotil AC whereas, all birds in the remaining treatments were vaccinated with the live F strain MG vaccine in drinking water as per manufacturer recommendation (0.8 mL PAC/Liter of drinking water- three days application) at 16 weeks of age. Moreover, treatments 3, 4, 5, and 6 were administered Pulmotil AC after by 3, 7, 14, and 21 days post vaccination respectively. Treatments are detailed in Table 1.

Table 1. Treatment allocation to experimental birds

Treatment	Vaccination with AviPro	Pulmotil Treatment in drinking water	Application of Pulmotil AC. No. of days post vaccinaation
1	None	None	None
2	Week 16	None	None
3	Week 16	Yes	3 days Post vaccination
4	Week 16	Yes	7 days Post vaccination
5	Week 16	Yes	14 days Post
6	Week 16	Yes	21 days Post

C. Isolation and Identification of MG

The following procedures were followed to isolate and identify MG and prepare Frey's Medium:

1. Fresh Yeast Extract Preparation

- 250g of baker's yeast were soaked in 1 liter of distilled water for 1 hour and then heated until boiling.
- After cooling, the suspension was distributed in tubes of 50 ml then centrifuged at 3000 xg for 20 minutes.
- The supernatants were collected and filtered through Whatman No. 1 filter paper and their pH was adjusted to 8 by using NaOH.
- The extracts were filtered through a 0.8µm filter paper then filter sterilized through a 0.22µm filter paper to remove any potential bacterial and/or fungal contamination.
- Aliquots of 15 ml of the sterile extracts were distributed in sterile cups and stored at -20 °C.

2. Inactivation of the Swine Serum

- Sterile swine serum (Gibco, Grand Island, N.Y. 14072, USA) was heat inactivated in a water bath at 55°C for 30 minutes. This treatment inactivates the complement system protein in the serum.
- Aliquots of 18 ml were distributed in sterile conical tubes and stored at -20°C.

3. Frey's Broth Preparation

- The selective broth was prepared as described by Frey *et al*. (1968).
- For the preparation of 150 ml of broth 3.37g of *Mycoplasma* broth base (Oxoid Ltd. Basingstoke, Hampshire, England), 375µl of 10% w/v phenol red and 425µl of 10% thallium acetate are mixed in 113.5 ml of distilled water.
- The mixture was boiled and pH was adjusted to 7.8 with 0.1N NaOH.
- After sterilization at 121°C and 20 psi for 15 minutes, the mixture was cooled and 15 ml of fresh yeast extract, 18 ml of heat inactivated swine serum, 750µl of penicillin containing 150000 I.U. and 2 ml of filter sterilized dextrose (0.225g/ml) are added to the mixture.
- The broth was distributed into sterile screw-capped tubes (5ml/tube).
- The pH was readjusted to 7.8 using sterile 0.1N NaOH.
- The tubes were ready for sample collection. It is worth noting that for highly contaminated samples it is recommended to raise the level of the thallium acetate concentration from 425µl to 750µl of 10% thallium acetate and 3 ml of 1% thallium acetate. Penicillin could be also raised to 450000 I.U. in 150 ml of Frey's broth.

4. Frey's Agar Preparation

The agar was prepared using the same materials and in their respective amounts. However, 1.5g of Bacto-agar (Difco Lab, Detroit, Michigan, USA) was added to the mixture of MG broth base, phenol red, and thallium acetate before boiling and sterilization. The mixture was allowed to cool till 55°C in a water bath. Fresh yeast extract, swine serum, dextrose and penicillin were then added to the mixture and the pH is adjusted to 7.8 with sterile 0.1N NaOH. Frey's agar is then poured in small sterile plates (10 ml/plate).

D. Sample Collection

- Tracheal swabs were collected from 10 randomly selected birds from each pen on at 3, 7, 14, 21, 28 and 35 days post Pulmotil treatment (dpp). These swabs were then vigorously shaken in tubes containing 5ml of Frey's broth, 2 ml placed in the incubator and 3 ml subjected to DNA extraction for the quantification of MG colony forming units by the aid of real time PCR (qPCR).
- Blood collection for seroconversion studies: Around 3 ml of blood were collected from the wing vein of all the birds in non-heparinized tubes. They were centrifuged for sera collection at 16, 19 and 22 weeks of age. The sera were used to assess the levels of anti-MG antibodies on different dates by using ELISA kits (IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine 04092, United States)

The blood was centrifuged for 10 minutes at 2000 rpm to collect sera.

E. Culture Method for the Detection of MG

Tubes containing tracheal suspensions (2 ml) were set in the incubator at 37°C

for 3-4 days or once the observation of phenol red color changed to orange. If the color changed to yellow, filter sterilization in 0.22μm pores of the substrate is re-cultured and incubated again to confirm the suspected results.

F. Q-PCR for the Quantification of MG in Frey's Broth Tracheal Suspensions

1. DNA Extraction of Frey's Broth Tracheal Suspensions

Tracheal samples were subjected to a temperature of 90°C for 10 minutes then to a freezing temperature of -80°C for DNA extraction.

2. Real Time PCR for Tracheal Swabs

The DNA extracted from the Frey's broth tracheal suspension was subjected to real time PCR (q-PCR) (i-Taq Bio-Rad) at 3, 7, 14, 21, 28 and 35 days post Pulmotil AC treatment.

The protocol of RT-PCR was conducted as follows:

DNA strands are denatured at 95°C; Annealed and extended at 60°C for 1 minute and for 40 cycles as per manufacturer's recommendations. The reagents that were used are detailed in Table 2.

Table 2. Reagents and Volumes for preparation of Real-Time PCR mixture

Reagent	**Volume**
iTaq	10μL
Probe	1μL
Forward Primer (MgC_2F)	1μL
Reverse Primer (MgC_2R)	1μL
Template DNA	3μL
PCR Water	4 μL
Total Volume	20μL

G. Seroconversion and ELISA

Seroconversion against MG was done by the enzyme linked immunosorbent assay (ELISA) as per the below protocol.

1. Preparation of Samples

- The test samples were diluted five hundred folds (1:500) with sample diluent prior to being assayed i.e 1 µl of sample in with 500 µl of diluent. The control groups are not diluted.
- The tips were changed for every sample.
- The samples were vortexed for 30 seconds before dilution, mixed and dispensed into the antigen coated plate.

2. Sera Dilution

- Plate charts for each plate was sketched accordingly for sample well and identification. Sera were allowed to come to 18-26°C, and then mixed via vortexing.
- Diluted 1/50; 200µl of dilution buffer was loaded into dilution plate, and then 4ul of each serum sample was added and mixed with dilution buffer 6 times.
- Diluted 1/10; 180µl of dilution buffer was loaded into a new dilution plate and 20µl of each step 1 diluted serum sample was added and mixed with dilution buffer 6 times.

3. ELISA

- The reagents were allowed to come to 18–26°C, and then mixed gently by inverting and swirling.

- Antigen-coated plate(s) were obtained and the samples recorded.
- A 100 µl of UNDILUTED Negative Control was dispensed into duplicate wells.
- A100 µl of UNDILUTED Positive Control was dispensed into duplicate wells.
- A100 µl of diluted sample was dispensed into appropriate wells.
- The mixture was incubated for 30 minutes (± 2 minutes) at 18–26°C to allow the binding between the protein antibodies and the wall coating antigens.
- Each well was washed with approximately 350 µl of distilled or deionized water 3 to 5 times to wash the excess unbound antibodies.
- A 100 µl of Conjugate was dispensed into each well. The conjugate targets the chicken IgG and it is labeled with peroxidase enzyme.
- The micro titer plate was incubated for 30 (± 2 minutes) minutes at 18–26°C.
- Repeat step 6.
- A 100 µl of TMB Substrate Solution was dispensed into each well. The conjugate peroxidase breaks the TMB substrate into a blue colored compound.
- The micro titer plate was incubated for 15 minutes (± 1 minute) at 18–26°C (in the dark).
- A100 µl of Stop Solution was dispensed into each well to stop the reaction.
- The absorbance values were recorded and measured at 650nm, A(650) using ELISA reader machine.
- **NOTE:** For the assay to be valid, the difference between the Positive Control mean and the Negative Control mean (PCx–NCx) should be greater than 0.075.

The Negative Control mean absorbance should be less than or equal to 0.150.

- The presence or absence of antibody to MG is determined by relating the A(650) value of the unknown to the Positive Control mean. The Positive Control is standardized and represents significant antibody levels to Mg in serum.
- The relative level of antibody in the sample is determined by calculating the sample to positive (S/P) ratio. Endpoint titers are calculated using the equation described in the calculations section.

H. DNA Sequencing of the MG Vaccine F-Strain

Upon receiving the AviPro® MGF, an aliquot of the vaccine suspension was subjected to DNA Extraction using the Qiagen DNA minikit (Qiagen GmBH, Hilden, Germany) and PCR amplification targeting the adhesin protein-coding gene (*mgc2*) (Grodio et al., 2008). The resulting amplicon was sequenced using the automated Sequencer 3100 Avant Genetic Analyzer- ABI PRISM instrument (Applied Biosystems, Hitachi) to confirm that it was an F strain of the experimental MG vaccine.

I. Experimental Period

The trial was completed in a period of one year after receiving the approval from the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB). All of the birds were marketed and revenues credited to the main investigator's research account.

J. Statistical Design and Analysis

The design of the trial was a complete randomized design with 6 treatments

and 18 birds per treatment. One-way ANOVA was used to compare means, followed by Tukey's test for mean separation using the proper procedures of SAS or SPSS V22.

CHAPTER IV

RESULTS AND DISCUSSION

A. DNA Sequencing of the MG Vaccine F-Strain

The PCR was performed successfully amplifying the *mgc*2 gene of AviPro MG vaccine strain. It resulted in the formation of a band of 227 bp in length as shown in Figure 4 (Grodio *et al*., 2008).

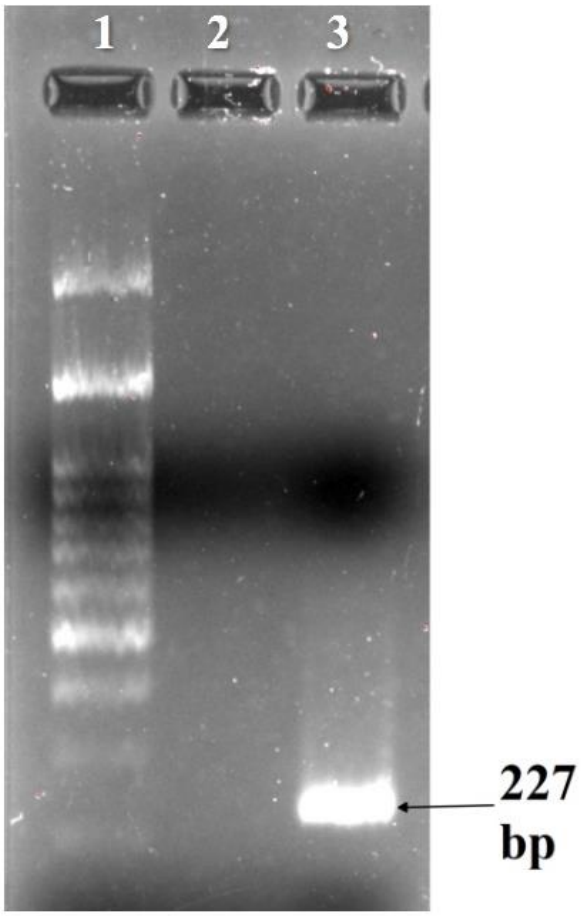

Fig. 4. AviPro mgc2 amplicon (Lane 3); Lane 1: 100 bp ladder; Lane 2: Negative control

The band resulting from the amplification of the *mgc2* adhesin-coding gene was sequenced and aligned to internationally reported *mgc*2 sequences using the National Center for Biotechnology Information (NCBI) BLAST function (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A 100% similarity to that of the F strain was reported.

B. Evaluation of MG colonization in the Trachea

Table 3 shows the percentage of MG positive swab samples collected from the breeder pullets at various days' post PAC treatment.

Table 3. Percentage of MG positive swab samples (culture) collected from the 16-week-old broiler breeder pullets

Treatment	Vaccinated	PAC administration	Percentage of positive swab samples at**:					
			3 dpp	7 dpp	14 dpp	21dpp	28 dpp	35 dpp
1	No	No	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
2	Yes	No	40^{b1}	$60^{b1,2}$	$70^{c2,3}$	100^{c3}	$75^{bc2,3}$	100^{c3}
3	Yes	3 dpv	0^{a1}	0^{a1}	0^{a1}	0^{a1}	50^{b2}	70^{bc2}
4	Yes	7 dpv	0^{a1}	0^{a1}	10^{ab1}	10^{a1}	62.5^{b2}	50^{b2}
5	Yes	14 dpv	0^{a1}	0^{a1}	33.3^{b2}	$50^{ab2,3}$	$50^{b2,3}$	80^{bc3}
6	Yes	21 dpv	0^{a1}	40^{b2}	100^{c3}	$70^{bc2,3}$	100^{c3}	100^{c3}

[a-c] Percentages in a column with different alphabetical superscripts are significantly different (P<0.05)

[1-3] Percentages in a row with different numerical superscripts are significantly different (P<0.05)

[*] dpv = days post vaccination

[**] dpp = days post PAC treatment

The absence of positive samples in the control treatment 1 indicates that the experiment was followed up regularly and the biosecurity measures were properly applied. Treatment 2 had a successful and progressive tracheal MG colonization where

it significantly increased from 40% at 3 days post PAC (dpp) treatment to 70% at 14 dpp, reaching a plateau that was maintained till the end of the trial. Treatments 3, 4, 5, and 6 were given Pulmotil®-AC at 3, 7, 14, and 21 days post vaccination (dpv), respectively. MGF in treatment 3 was absent from tracheal swab samples until 21dpp inclusive. It significantly increased, however, to 50% and 70% at 28 and 35 dpp, respectively. This is probably an indication of a prominent anti-MG effect of PAC in this treatment. Treatment 4 had results comparable to those of treatment 3 where the MG colonization plateau was reached at 28 dpp. MG recolonization rate in treatment 5 was more active in comparison to treatment 4 where the plateau was reached at 21 dpp. Treatment 6 showed the highest and earliest recolonization pattern among all PAC-treated groups where 40% value was recorded at only 7 dpp with a plateau reached 14 days dpp onwards. Although the recolonization pace varied among the experimental treatments, all treatments showed an effective MGF recolonization at different days after the treatment with PAC was discontinued. In fact, these results indicated that the recolonization efficiency improved as the PAC treatment was delayed. This was reflected by the rapid MG recolonization rate in birds of treatment 6, as compared to all other treatments, that had a late PAC administration after vaccination (21 dpv).

Table 4 shows the MG CFU count (/mL) of Frey's broth tracheal swab suspensions, as concluded from the amount of MG-DNA determined by q-PCR analysis at different days post PAC treatment.

Table 4. MG CFU count (/mL) of Frey's broth tracheal swab suspensions (q-PCR)

Treatment	**Vaccinated**	**PAC trt.***	**CFU count (x 10^3)/ ml Frey's broth at****					
			3 dpp	**7 dpp**	**14 dpp**	**21dpp**	**28 dpp**	**35 dpp**
1	No	No	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
2	Yes	No	27.2^{b}	12.5^{ab}	16.6^{b}	6.0^{b}	82.6^{b}	7616.4^{b}
3	Yes	3 dpv	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	1.7^{a}	0.0^{a}
4	Yes	7 dpv	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	4.8^{a}	18.9^{a}
5	Yes	14 dpv	0.0^{a}	0.0^{a}	5.4^{ab}	0.0^{a}	18.5^{a}	2321.6^{ab}
6	Yes	21 dpv	5.6^{a}	14.5^{b}	6.9^{ab}	5.6^{a}	18.2^{a}	2839.3^{ab}
SEM (x10^3)			1.76	1.46	1.72	4.03	11.18	744.42

$^{a-b}$ Percentages in a column with different alphabetical superscripts are significantly different ($P<0.05$)

*dpv = days post vaccination

**dpp = days post PAC treatment

The absence of MG count in treatment 1 indicates again the success of the implementation of biosecurity measures in this experiment.

During the first 3 days following PAC adminstration for treatments 3, 4, 5 and 6, it was obvious that the only significant CFU count was recorded for the vaccinated, PAC-deprived treatment 2. Tracheal MG recolonization was consistently detected in treatments 2 and 6 only, at 3 days onwards. As of 28 dpp, MG recolonization of the trachea was restored for treatments 4 and 5, indicating a complete recovery of MGF following PAC treatment at the indicated days.

There was no significant difference among treatments 2, 5 and 6 at 35 dpp, showing significantly higher MG counts in comparison to the other treatments. This means that MG was recovered again at this date and had enough time to replicate and recolonize the tracheas. Treatment 3 showed a hindered recolonization pattern after the administration of PAC at 3 dpv. This was reflected by the CFU count that remained close to zero during the whole experimental run and confirmed the results previously discussed in Table 3.

C. Sera Titers to MG

Table 5 shows the sera titers to MG of 6 treatments at different dates post vaccination and PAC treatment.

Table 5. Sera titers to MG of birds vaccinated at 16 weeks of age at various days post vaccination with AviPro

Treatment	**Vaccinated**	**Pulmotil trt.***	**Sera Titers at****			
			0 dpv	**7 dpv**	**28 dpv**	**49 dpv**
1	No	No	<300	<300	<300[a]	<300[a]
2	Yes	No	<300	<300	535[b]	1128[ab]
3	Yes	3 dpv	<300	<300	431[ab]	494[a]
4	Yes	7 dpv	<300	<300	<300[a]	497[a]
5	Yes	14 dpv	<300	<300	<300[a]	788[a]
6	Yes	21 dpv	<300	<300	345[ab]	2160[b]
SEM			18.4	8.9	25.9	119.3

[a-c] Percentages in a column with different alphabetical superscripts are significantly different (P<0.05)

*dpv = days post vaccination

**dpp = days post PAC treatment

On the first day of vaccination, the sera titers to MG were below the detectable level of the ELISA kit for all the treatments, indicating the absence of MG titers in birds at the beginning of the experiment (16 weeks of age). MG titers were still low and insignificantly different among various treatments at 7 days post vaccination, revealing the absence of detectable immune response to the vaccine. The significant differences started to appear as of the 28th days post vaccination for treatments 2, 3, and 6, yet the titers were all below 1000. At 49 days post vaccination, the highest sera titers were recorded for groups 2 and 6. Treatment 2 titers peaked at the same date, namely 49 days post vaccination, recording a titer of 1128. The significant peaking of treatment 6 titers

at 49 dpv further reflects the successful colonization rate in the trachea of birds belonging to this treatment as indicated previously in Tables 3 and 4.

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

Tilmicosin, a semi-synthetic form of macrolide; interferes with protein synthesis by reversibly binding to the 50S subunit of the ribosome; preferentially to the 23S rRNA of the 50S subunit; inhibiting translocation that is required for the elongation of peptide chain via their attachment to the donor site. It is essentially effective against *Mycoplasma* spp., *Pasteurella* spp., and distinct Gram-positive organisms including Gram-positive anaerobic species and Gram-negative respiratory pathogens.

Tilmicosin behaves as a bacteriostatic but at high concentrations it may possess bactericidal activity. Tilmicosin and macrolide have been suggested to act synergistically with the host immune system; augmenting phagocytosis of bacteria (Ramadan, 2018). As per above results, all treatments vaccinated with AviPro® MGF showed recolonization after the treatment with Pulmotil® AC was discontinued, which indicates that the Mycoplasma strain of AviPro® MGF endures Pulmotil® AC treatment. The results recorded for treatment 6, in this study, indicated the highest recolonization rate and pace among PAC treated groups. Treatment 6 showed an early start of MG recolonization at 3 days and complete recolonization at the 14th day post Pulmotil® AC treatment. Figures revealed the proportionality between the interval of PAC application post vaccination and the recolonization rate. Therefore, and for a successful combination of live vaccine and antibiotics to be recommended, it is more preferable to vaccinate broiler breeder pullets at 16 weeks of age with AviPro® MGF live vaccine followed by PAC treatment 21 days after vaccination. This will ensure successful treatment of AviPro® MGF vaccinated birds with Pulmotil® AC without significantly

affecting the MG tracheal colonization by the vaccine strain of AviPro® MGF.

It is also recommended in the future to evaluate the humoral immunity response of MG in a more specific way, namely evaluating the IgA levels in the mucosal surfaces of the respiratory system following the vaccination with AviPro.

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