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Study of Interaction Between AviPro® *Mycoplasma gallisepticum* (MGF) and Pulmotil®-AC in Broiler Breeder Pullets

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Abstract

This study aimed at the determination of 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. A total of 108 sixteen-weeks-old breeder pullets of the ROSS 308 strain were subdivided equally into 6 groups. Pullets of group 1 remained unvaccinated and untreated with PAC. Birds of groups 3, 4, 5 and 6 were vaccinated with AviPro® MGF at 16 weeks of age and treated with PAC at 3, 7, 14, and 21 days post-vaccination respectively. Group 2 was kept as the vaccinated non-treated group. The pullets were tested for the presence of MGF strain in the trachea at different days after PAC-treatment completion. All PAC-treated groups showed tracheal MG recolonization after the treatment was discontinued. The percentage of positive tracheal MG swab cultures was consistently higher in group 6, reaching a plateau at 14 days post PAC treatment (100%, $P<0.05$). qPCR implied-tracheal MG counts indicated better recolonization efficiency for birds of groups 5 and 6 reaching up to 2322×10^3 and 2839×10^3 cfu/ml of broth, respectively, at 35 days post PAC treatment. Moreover, Group 6 showed the significantly highest titer to MG, recording a value of 2160, followed by the vaccinated untreated Group 2 (1128). For a successful application of live MG vaccine/antibiotics combination, it is recommended to delay PAC treatment 21 days after the vaccination of breeder pullets with AviPro® MGF.

Keywords: *Mycoplasma gallisepticum*; AviPro MGF; Pulmotil AC; Pullets; qPCR; ELISA

Introduction

Mycoplasma gallisepticum (MG) infection in poultry is the etiologic agent that is responsible for the development of chronic respiratory disease (CRD). MG predisposes broilers, layers and breeders to severe secondary infections such as colibacillosis. Moreover, MG infection affects egg quality and production, and results in reduced feed efficiency, condemnation and downgrading of broilers carcasses at slaughter because of air sacculitis. Therefore, MG infection is considered as one of the most economically significant diseases in poultry [1,2]. Ideally, MG-infected flock should be eradicated in order to contain the circulation of the pathogen; however, in many countries, the control of MG infection in poultry necessitates the interference with antibiotics [3]. Abd El Hamid et al. [4] mentioned that tiamulin was found to be the most effective drug against 10 MG field isolates with minimal inhibitory concentration (MIC) of 0.0125-0.4 µg/ml, followed by doxycycline (MIC: 0.003-0.4 µg/ml), then tylosin (MIC of 0.025-0.4 µg/ml), Enrofloxacin (MIC of 0.0125-0.1 µg/ml), Ciprofloxacin (MIC of 0.2-1.6 µg/ml) and finally Erythromycin (MIC of 3.2-6.4 µg/ml). Nevertheless, there is abundance in literature reporting the emergence of MG resistance to various antibiotics. Alun and Ching [5] and Pakpinyo and Sasipreeyajan [6] reported strong resistance to erythromycin, while Ellakany et al. [7] found that MG field isolates from Egypt were highly sensitive to tiamulin, tylosin, and moderately sensitive to enrofloxacin. Resistance to tylosin, the drug of preference, also increased in the last decades as reported by Hannan et al. [8] and Valks and Burch [9].

On the other hand, the prevention of MG infection in poultry necessitates the application of strict biosecurity measures including 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 MG mainly colonizes the respiratory mucosal linings, thus hindering the accessibility of IgG to the colonizing pathogen. 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 [10,11].

This preliminary study combines the use of tilmicosin (Pulmotil® AC), and AviPro® MGF (*Mycoplasma gallisepticum* vaccine, live culture) in order to develop a sound protocol for the control of *Mycoplasma gallisepticum* (MG) in breeder pullets. In addition, the aim is to determine the number of days that Pulmotil® AC should be separated from the vaccination with AviPro® MGF to avoid any interference with the vaccine and to study the MGF re-population pattern in the vaccinated birds after the treatment is completed.

Materials and Methods

Birds and housings

This experiment was approved by the Institutional Animal Care and use Committee (IACUC) of the American University of Beirut. It was conducted at the research facilities of the University in the Beqaa region where equipped poultry houses are available. A total of 108 sixteen-week-old pullets, of the Ross 308 strain, were equally subdivided into six groups of 18 birds each. Birds were given water ad libitum and feed as per the Breeder Manual recommendations provided by the breeding company. At arrival, swab samples were taken from the trachea of 10 birds to confirm that the birds were MG free using Frey's culturing method [12] and Polymerase Chain Reaction [13].

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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 a 267 bp fragment of the adhesin protein-coding gene (mgc2) [14]. The resulting amplicon was sequenced using the automated Sequencer 3100 Avant Genetic Analyzer- ABI PRISM instrument (Applied Biosystems, Hitachi) to confirm the F strain identity of the experimental MG vaccine.

Treatments

Birds were assigned to different treatments, including vaccination via drinking water with the AviPro® MGF, and administration of Pulmotil® AC as indicated in Table 1.

Evaluation of MG colonization in the trachea

A total of 10 individual tracheal swabs were taken at 6 different dates post-treatment namely 3, 7, 14, 21, 28 and 35 days. Swab rubbings were collected in 5 mL of Frey's Broth [12] which were then equally divided into two separate sterile tubes (2.5 mL/tube) and tested for the presence of MGF. The quantitation of MG colony forming units was performed using culture [12] and real time PCR (qPCR) according to Grodio et al. [13] as detailed below.

Determination of the frequency of positive MG tracheal swab samples by Frey's Broth culture: The first portion of tracheal rubbings in Frey's broth (2.5 mL/tube; 10 samples per group) was incubated at 37°C for a period of one week. Samples were considered positive whenever the broth color turned orange, as a result of sugar fermentation and pH drop, within a range of three-seven days [2].

Real time PCR assays: DNA was extracted by incubating the second set of tubes containing Frey's Broth tracheal rubbings in a water bath at 95°C for 10 minutes and then placed on ice for 10 min [15]. The same procedure was adopted to extract the DNA of the AviPro MGF vaccine suspension with an initial concentration of 15 µg/µL corresponding to 10^6 MG CFU/mL Frey's Broth (Stock). Fresh ten folds dilution of the stock DNA was prepared and quantitated using Nanodrop 2000c (Thermo Scientific, USA).

The 20 µl reaction was performed in CFX96 1000c (BioRad laboratories, 2000 Alfred Nobel Drive, CA, USA). The q-PCR reaction utilized 10 µl TaqMan iTaq PCR Mix (BioRad laboratories, 2000 Alfred Nobel Drive, CA, USA), 1 µl of each of 5 pmol forward and reverse primers and the probe [13], 4 µL DNase-free water and 3 µl DNA of samples or standards. Samples were run in duplicate. Cycling parameters were 95°C for 3 mins and 40 cycles of 95°C for 10 sec and 60°C for 30 sec and the automatic threshold settings were used in analysis of samples. Samples were considered positive for *M. gallisepticum* if the cycle threshold (Ct) value was less than 40. A PCR run, targeting a 303 bp fragment of the mgc2 gene, was considered valid whenever the reaction was 99.2-100% efficient, and R^2 ranged between 0.996 and 0.999. MG colony forming units (CFU) of each sample were concluded then from the amount of DNA quantitated by the q-PCR assay. It is worth noting that the qPCR assay was able to detect down to 1.5 ng of DNA, equivalent to 100 MG CFU per mL of broth.

Blood collection for seroconversion studies

Blood samples were collected from all the birds (20/group) before vaccination, and at 16, 19 and 22 weeks of age for MG seroconversion. Briefly, 3 ml of blood were collected from the wing vein of the bird in non-heparinized tubes. Blood was allowed to settle for one hour at room temperature and then centrifuged for 10 minutes at 2000 rpm to collect the sera that were later preserved at -20°C until further analysis. ELISA kits (IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine 04092, United States) were used to assess the levels of anti-MG sera titers at the above indicated days.

Statistical design and analyses

The design of the trial was a complete randomized design with 6 treatments and 18 birds per treatment. One way ANOVA was performed, followed by Tukey's test for mean comparison using the proper procedures of SAS, 2008 [16].

Results and Discussion

DNA sequencing of the MG vaccine F-strain

The PCR was performed successfully, amplifying the mgc2 gene of AviPro MG vaccine strain. It resulted in the formation of a band of 267 bp in length as shown in Figure 1. As per the work of Liu et al. [14] in developing a PCR test for the diagnosis and typing of MG, the selected primers target a 267 bp region of the cytadhesion gene (mgc2) of the F strain specifically. Consequently, the current PCR outcome offers an additional confirmation that AviPro MG vaccine is of strain F. In the same context, it is worth noting that the selected primers target a 497 bp region in R and ts-11 strains, 437 bp region in 6/85 and S6 strains, and 410 bp region in the K strain [14].

The band resulting from the amplification of the mgc2 adhesin-coding gene was sequenced and aligned to internationally reported mgc2 sequences using the National Center for Biotechnology Information (NCBI) BLAST function [17]. A range of 97-100% similarity to that of reported F strain was revealed while it didn't exceed 96% to that of K strain, and even lesser in comparison to the rest, including R strain (less than 92%). These findings not only confirm the strain of AviPro MG, but also emphasize the high frequency of phase variation of mgc2 cytadhesin gene among MG strains which makes it a target gene preference in any diagnostic or evolutionary studies, escape mutation and pathogenesis [18-20].

Group	Vaccination with AviPro® MGF	Pulmotil® AC Treatment in drinking water	Days of application of the Medication
1	-	-	-
2	Week 16	-	-
3	Week 16	+	3 days Post vaccination and as per the manufacturer recommendation (0.8 mL PAC/Liter of drinking water-three days application)
4	Week 16	+	7 days Post vaccination and as per the manufacturer recommendation (0.8 mL PAC/Liter of drinking water- three days application)
5	Week 16	+	14 days Post vaccination and as per the manufacturer recommendation (0.8 mL PAC/Liter of drinking water- three days application)
6	Week 16	+	21 days Post vaccination and as per the manufacturer recommendation (0.8 mL PAC/Liter of drinking water- three days application)

Table 1: Treatments allocation with different designated days of application of medication.

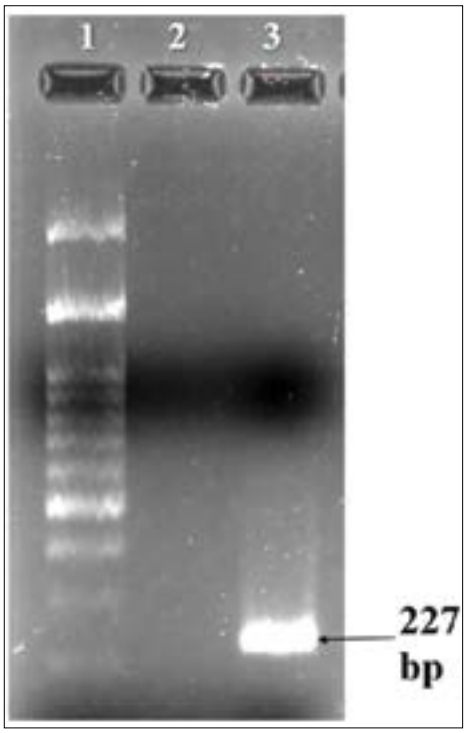

Figure 1: AviPro mgc2 amplicon (Lane 3). Lane 1: 100 bp ladder; Lane 2: Negative control.

Evaluation of MG colonization in the trachea

Table 2 shows the percentage of MG positive swab samples collected from the breeder pullets at different days post PAC treatment. Knowing that MG can be readily transmitted from bird to bird resulting in high infection and disease prevalence within flocks [2,21], the absence of positive samples in the control Group 1 indicates that the experiment was followed up regularly and the biosecurity measures were properly applied. Birds of Group 2 had a successful and progressive tracheal MG colonization where it significantly increased from 40% at 3 days post PAC treatment (dpp) to 70% at 14 dpp, reaching a plateau that was maintained till the end of the trial. The growth pattern of this avirulent MG strain in the respiratory tissue of poultry is well documented in literature; MG needs 1-4 weeks to colonize the mucosal linings depending on the virulence of the strain [2,22,23]. Groups 3, 4, 5, and 6 were given Pulmotil®-AC at 3, 7, 14, and 21 days post vaccination (dpv), respectively. MGF in Group 3 was absent from tracheal swab samples until 21 dpp 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 group. Group 4 had results comparable to those of Group 3 where the MG colonization plateau was reached at 28 dpp. MG recolonization rate in group 5 was more active in comparison to group 4 where the plateau was reached at 21 dpp. Group 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 groups, all groups 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 Group 6, as compared to all other groups, that had a late PAC administration after vaccination (21 dpv).

Figures shown in Table 3 represent a mirror image and a further confirmation of the results obtained in Table 2. Table 3 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.

The absence of MG count in Group 1 indicates again the success of the implementation of biosecurity measures in this experiment. During the first 3 days following PAC treatment for Groups 3, 4, 5 and 6, it was obvious that the only significant CFU count was recorded for the vaccinated, non-PAC treated Group 2. Tracheal MG recolonization was consistently detected in groups 2 and 6 only, at 3 days onwards. As of 28 dpp, MG recolonization of the trachea was restored for groups 4 and 5, indicating a complete recovery of MGF following PAC treatment at the indicated days.

There was no significant difference among groups 2, 5 and 6 at 35 dpp, showing significantly higher MG counts in comparison to the other groups. This means that MG was recovered again at this date and had enough time to replicate and recolonize the tracheas. Group 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.

Regardless of the recolonization pattern observed in this study, the fact that AviPro MGF strain endured tilmicosin treatment in all the experimental groups is remarkable. Tilmicosin is currently one of the most efficient drugs for the control of MG in poultry [24,25]. For certain MG isolates, the Minimal Inhibitory Concentration (MIC) of tilmicosin is proven to be, by far, lower than that of other drugs such as doxycycline, tetracyclin, enrofloxacin, gentamycin and even tylosin [26,27]. The same applies for the Mutant Prevention Concentration (MPC) and MPC/MIC ratio of this macrolide [27]. Nevertheless, AviPro MGF strain in this study endured a three-day tilmicosin treatment thus paved the way towards adopting a new model for the protection of breeder pullets. Although previous researchers [28], worked on a combined live MG vaccination/tylosin administration model for layers, the antibiotic used at 50 g/ton of feed affected negatively the 6/85 vaccine strain growth as it was reflected by low bird seroconversion frequency MG at various dates. Consequently, the results obtained in the current study were promising in regards to the adoption of a successful combination of live vaccine/antibiotic application for the control of MG in breeders. Having in mind that macrolides are protein synthesis inhibitors and work by binding to the 50S subunit of the MG ribosome [29], the mechanism of resistance to tilmicosin adopted for this vaccine strain remains proprietary information to the manufacturer. Suggested mechanisms of resistance involves, but not limited to: 1) the ability of MG to produce specific enzymes such as rRNA methylases that can add one or two methyl groups to the same adenine residue, thus reducing the binding of the macrolides to the ribosomal target site [30,31], and 2) Mutational events such as the substitution of specific nucleotides in genes coding for 23S rRNA (Domain V) [32].

Sera titers to MG

Table 4 shows the sera titers to MG of 6 Groups at different dates post vaccination and 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 groups, indicating the absence of MG titers in birds at the beginning of the experiment (16 weeks of age). MG titers were still

Group	Vaccinated	PAC trt.*	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

Table 2: Percentage of MG positive swab samples (culture) collected from the 16 week-old broiler breeder pullets.

Group	Vaccinated	PAC trt.*	qPCR implied-CFU count (× 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 (× 10^3)			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

Table 3: MG CFU count (/mL) of Frey's broth tracheal swab suspensions as implied from q-PCR assays.

Group	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

Table 4: Sera titers to MG of birds vaccinated at 16 weeks of age at various days post vaccination with AviPro.

low and insignificantly different among various groups 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 groups 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. Group 2 titers peaked at the same date, namely 49 days post vaccination, recording a titer of 1128. The significant peaking of Group 6 titers at 49 dpv further reflects the successful colonization rate in the trachea of birds belonging to this group as indicated previously in Tables 2 and 3.

It is generally accepted that MG live vaccine strains such as ts-11, F and 6/85 do not induce high antibody titers [2,33], nevertheless, AviPro MGF offers a dual protection mechanism, either alone or in combination with tilmicosin treatment (Groups 2 and 6, respectively) as demonstrated in this study. This can be explained by the ability of the live AviPro MGF vaccine to exert its protective effect against field strains, not only by competitive exclusion, as reflected by the tracheal colonization rate, but also by enhancing protective humoral response as revealed by the ELISA titers obtained in this trial.

Conclusion

For a successful combination of live vaccine and antibiotics to be recommended, it is more preferable to separate PAC treatment 21 days after the administration of AviPro® MGF live vaccine to broiler breeder pullets. This will ensure efficient treatment of AviPro® MGF vaccinated birds with Pulmotil® AC while preserving the vaccine potency.

Conflict of Interest

The authors declare no conflict of interest.

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