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Serological detection of *Mycoplasma gallisepticum* infection on broiler chickens treated with Antibiotic , probiotic and Glycyrrhizic acid

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Abstract

This study conducted to estimated the effect of probiotic, Glycyrrhizic Acid and antibiotic to reduce the effect of Mycoplasma gallisepticum (MG) infection in broilers chickens by measuring immune response against MG by ELISA and roles of this treatment in reducing clinical signs and gross lesions for this purpose The experimental groups (30 chicks in each group) of the study include: Group1 (G1): control negative group was not received any treatment. Group2 (G2): Control positive chicks were infected by *Mycoplasma gallisepticum* strain (MG) at dose 0.2 ml from 10^6 CFU of MG, Group3 (G3): 30 chicks were received two types ND vaccine at first day with MG infection at 5th day the same dose as before. Group4 (G4): chicks were received MG infection at the same dose as before with probiotic at dose 2 $\times 10^{11}$ CFU / kg. Group 5 (G5): chicks were received MG infection at above dose with Glycyrrhizic acid at of dose 1 ml contain (165mg) / litter of D.W- orally with drinking water. Group6 (G6): chicks were received MG infection at above dose with tylosin at dose 1 gm/ 2 litter of D.W- orally with drinking water. The blood samples were collected from wing vein at 5th, 10th, 16th, 26th, and 35th day for immunological tests from 5 chicks of each group The MG antibody titers at 10th day in group 1 was recorded the lower MG antibody titer than other groups. While, the group 2 showed the higher titer level than other groups. In the group 3, 4, and 5 were displayed a significantly moderate MG titer level. this result indicated that this treatment enhancing resistance the MG infection through role the probiotic in the decrease the lung injury by reducing MG colonization and pro-inflammatory cytokines and improving the role of microbiota in the MG infection.

I. Introduction

Mycoplasma gallisepticum (MG) is one of the important pathogens in birds. It causes economic losses because chronic respiratory disease occurance. This organism can invade the immune system, resulting in several systemic damage **(1)**. *Mycoplasma gallisepticum* is a prominent poultry disease that has caused serious economic losses for the poultry industry **(2)**. *Mycoplasma gallisepticum* in broiler chicken flocks caused serious invasion of the air sacs, trachea, heart,

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lungs, and liver and caused catarrhal **(**3**)**. *Mycoplasma gallisepticum* infected broiler chicks showed depression, acute conjunctivitis, ruffled feathers, sneezing, coughing, and dyspnea, as well as the typical voices heard on farms throughout the sickness phase (3; **4**). Glycyrrhizic acid (GA), the primary bioactive compound has been as anti-inflammatory, antioxidant, and immunomodulatory **(4)**. Glycyrrhizic acid additives for broilers improved feed efficiency, genes expression, reduced bacterial activity **(5)**. Glycyrrhizic acid inhibited the protein expression of inflammatory factors **(6)**. Glycyrrhizic acid can be decreased the morbidity, mortality, air sac lesion when the infection with MG in broilers and increased feed conversion rate and weight gain **(7)**.The gastrointestinal microbiota is important supplements for immune modulation in chickens. The Probiotics decreased inflammation, oxidative damage, and respiratory distress, such as *Bifidobacterium* and *Lactobacillus*, which supporting immune system in the respiratory tract **(6).** Probiotics are commonly used to increase the growth and resistance of the diseases in chickens **(8)**. The supplementation contained *L. salivarius*, which reduced from the ability of MG to induce tracheal inflammation **(8).** *Bacillus subtilis* orally contribute in the resistance to the respiratory infection of the chickens **(9)**. Therefore, the strategies for regulation of microbiota of intestine are important in control many diseases, metabolic and inflammatory disorders **(10)**. Study the effect of probiotic, Glycyrrhizic Acid and antibiotic to reduce the effect of MG infection in broilers chickens. Antibody detection is the main diagnostic technique to examine the chikens for *Mycoplasma* infections **(11)**. Enzyme-linked immunosorbent assay (ELISA) is widely used in MG diagnosis because of its high sensitivity **(12)**. ELISAs were developed for the detection of *M. gallisepticum*, *M. synoviae*, and other species **(13)**.

II. Materials and Methods

Preparation of poultry house and managements

The College of Veterinary Medicine, University of Baghdad unite of poultry diseases hosted the experiments. The experimental house underwent cleaning, formalin disinfection, and another wash before the experiment commenced. Utilizing brooders, the temperature was managed. A litter of wood shavings that was 10 cm deep blanketed the ground. Each group faced the same breeding management. We cleaned and sterilized the utensils and feeders. Rations in full and tap water were used**.**

Preparation the inoculum for the field strain of *Mycoplasma gallisepticum*.

According to **Yagihashi and Tajima (14)** used PPLO broth and agar media were used in this study. *Mycoplasma gallisepticum* were grown through 24-48 hours at 37°C in the broth medium, and the stocks of growth were preserved at -20 °C. PPLO broth was diluted to reach bacteria concentration about 10^4 - 10^6 colony-forming units (cfu)/ml for chicken inoculation. These were made by making ten-fold dilutions of culture in *Mycoplasma* broth and plating, drop or 20 µl from each dilution on *Mycoplasma* agar plate; then plates were incubated with 5% CO_2 at 37°C. The *Mycoplasma* colonies appeared on agar plates and daily showed by dissecting microscope to evaluate with first five days, then every two days for up to four weeks. The optimum counts ranged from 30 to 300 colonies for each drop. **(15)**. The micropipette intratracheal route inoculated each chick with 0.2 ml of diluted culture from dilution 10^6 colony-forming units per for G2, G3, G4, G5, G6, and G7, respectively.

The Experiment

Broiler chicks (No. 210) of the breed (**Ross 308 Broiler chicks**) from the Al-Halafawi hatchery- Babil Al-Mahawil were used in this experiment from 1 to 35 days in order to expermintal study. The experimental groups of the study include:

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1-Group1 (G1): control negative group, 30 chicks were not received any treatment.
2-Group2 (G2): control positive included 30 chicks were infected at 5th day by MG strain at dose 0.2 ml from 10^6 CFU of MG with accession number: OR784572.1.and isolated by **Alhamza (2025)** by intra tracheal route, the dose was selected according to **(Ali,2019).**
3-Group3 (G3): 30 chicks were received two types ND vaccine at first day with MG infection at 5th day the same dose as before.
Group4 (G4): 30 chicks were received MG infection at 5th day the same dose as before with probiotic (Poultry star®) at dose 0.5 gm / 1 kg of feed through 5-15th from old.
Group 5 (G5): 30 chicks were received MG infection at 5th day the same dose as before with Glycyrrhizic Acid (CG-Herbs®) at dose 1 ml contain (165mg) / litter of D.W- orally by drinking water through 5-15th from old.
Group6(G6):30 chicks were received MG infection at 5th day the same dose as before with tylosin (Tylodad ®) at dose 1 g/ 2 litter of D.W- orally by drinking water through 5-15th from old.

Samples collections
A-Blood samples: the blood samples were collected from wing vein for all groups, at 5th, 10th, 16th, 26th, and 35th day for immunological tests from 5 chicks of each group.
B- Clinical signs, gross lesions post infection with MG.

MG Antibody ELIZA Kit
The antibodies of MG were estimated according kit instruction of SunLong Biotech, China
Statistical analysis

Data analysis was carried out by usi ng SPSS (IBM- version 20, USA). The one-way ANOVA were used to evaluate the significant differences between the means at $P \leq 0.05$ for determining statistical significance.

III. Result and Discussion

The result showed that one of the important clinical signs was foamy and watery ocular discharge showed in the infected chicken by MG. The upper respiratory tract with conjunctival inflammation often reflected in chronic respiratory disease (CRD) in chicken (Figure 1).

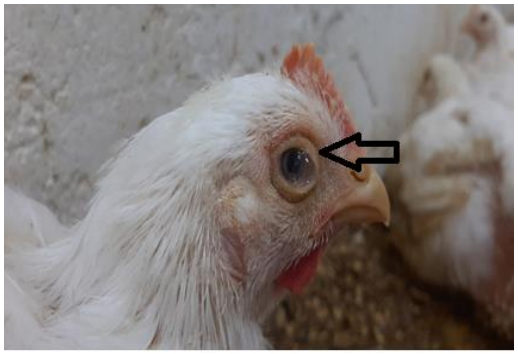

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Fig. 1 showed foamy watery eyes of chick with MG infection in G2

While the pathological lesions assessment of the air sacs, liver, and heart cross ways the studied experimental chicks showed clear changes. In Group 1 (G1), the negative control group, the air sacs appeared normal, without any noticeable lesions or pathological variations. However, in Group 2 (MG infected), which used as positive control, fibrinous exudate was detected in the liver, heart, and air sacs (Figures 2). A similar pattern was seen in Group 3 (MG - NDV), where chicks were vaccinated with Newcastle Disease virus (NDV) and then infected with MG; fibrinous exudates were again evident in the liver, heart, and air sacs (Figures 3).

In Group 4 (MG - NDV - Probiotic), which received NDV vaccination, MG infection, and probiotic supplementation, the air sacs showed a cloudy appearance along with notable thickening (Figures 4). Group 5 (MG - NDV - Glycyrrhizic Acid), treated with glycyrrhizic acid with NDV vaccination and MG infection, also confirmed a cloudy appearance of the air sacs, indicating moderate pathological lesions changes (Figure 5). Also, Group 6 (MG - NDV - Tylosin), which received tylosin drugs, presented a cloudy appearance in the air sacs, reflecting some improvement when compared to the untreated infected groups (Figure 6). Generally, fibrinous exudates were detected infected groups with MG only, while if chicks were treated with probiotics, glycyrrhizic acid, or tylosin appeared to slightly reduce the severity of the observed lesions.

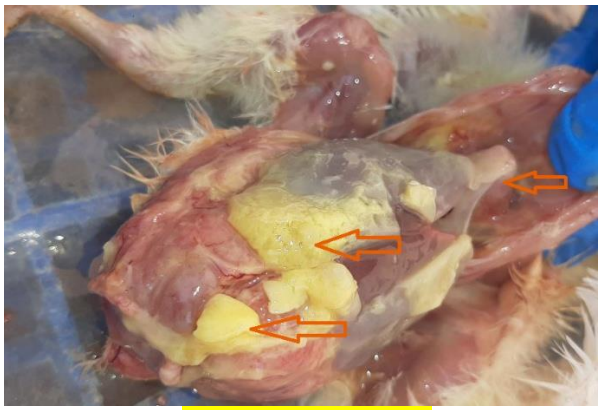

Fig. 2 Presence fibrinous exudate and caseated materials in liver, heart and air sac in G2

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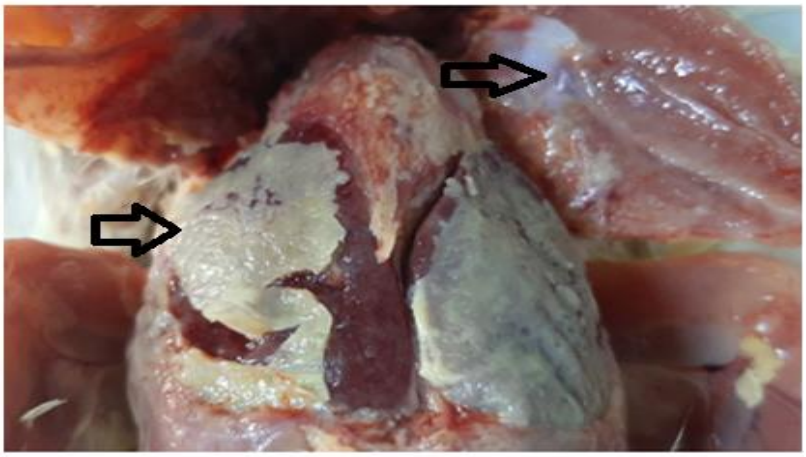

Fig. 3. Presence fibrinous exudate and caseated materials in liver, heart and air sac in G3

Fig. 4. Air sac appearance cloudy in G4

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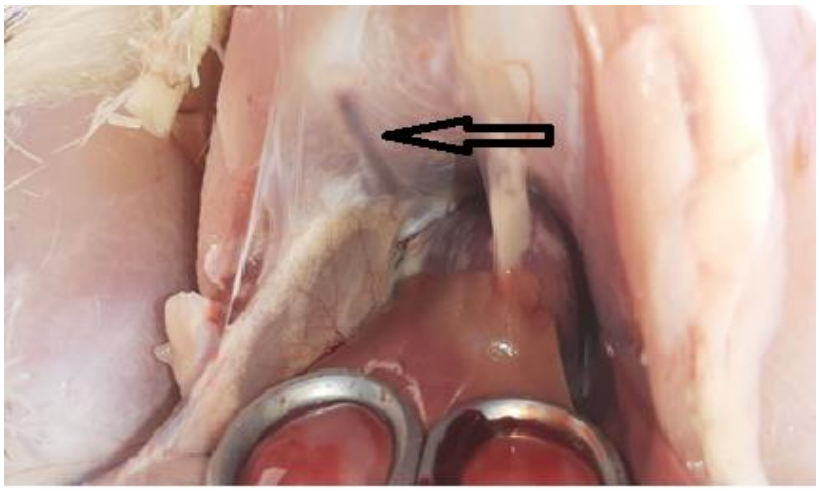

Fig.5.Air sac appearance cloudy and thickening in G5

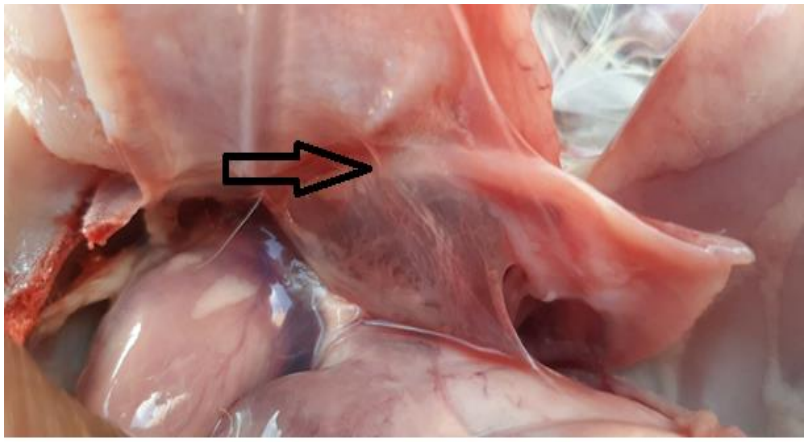

Fig.6. Air sac appearance cloudy in G6

The pathological lesion results were showed that MG infection caused significant lesions, included fibrinous exudate in the liver, heart, and air sacs, consistent with previous descriptions of chronic respiratory disease in poultry **(18)**. The marked pathological changes showed in the positive control chicks support findings that MG mostly infects the respiratory tract and spread systemically, particularly when secondary bacterial infections or immune suppression are complicated **(19)**. Use of probiotics in Group 4 markedly diminished the severity of lesions, as designated by cloudy but less fibrinous air sacs. This supports the results that probiotics can modulate the immune system, increase mucosal defenses and dropping the influence of respiratory infections **(20)**.

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Probiotics likely enhanced communication along the gut-lung axis, boosting overall resistance to respiratory pathogens. Similarly, glycyrrhizic acid use in Group 5 showed moderate improvements in air sac appearance. Known for its anti-inflammatory and immunomodulatory effects, glycyrrhizic acid has been reported to inhibit pathogen replication and ease inflammatory responses **(21)**. The cloudy air sacs suggest it provided some protection against MG progression, though it was not completely effective. Treatment with tylosin in Group 6 improved lesion severity compared to untreated infected groups, seen through cloudy air sacs and reduced fibrinous exudate. Tylosin, a macrolide antibiotic, has been commonly used to manage MG infections and is known to effectively reduce related clinical signs and lesions **(22)**. However, the incomplete resolution of lesions may point to emerging antibiotic resistance or less-than-ideal treatment conditions. In summary, while probiotics, glycyrrhizic acid, and tylosin each offered some protection against MG -induced lesions, none completely resolved the damage. This underscores the complexity of controlling chronic respiratory disease in poultry and suggests that a combination of vaccination, antibiotic use, and immune system support will be necessary for best results.

The result showed the MG antibody titers in the six chicken groups at three time periods of experiment 10th, 26th, and 35th days, at 10th day group 1 (Control Negative) was recorded a lower MG antibody titers (359.75 ± 52.1) than other groups, while the group 2 (MG infection) showed a higher titer level (1281.5 ± 77.6) than other groups and group 3 (MG infection with ND vaccine) confirmed the highest MG antibody titer (1347.75 ± 180.1) at 10th day in the experiment. Based on the results at 26th day and at 35th day from experiment, group 2 (MG infection) and group 3 (MG infection with ND vaccine) were continued to increase in titers than other groups, while group 1 (Control Negative) was exhibited a decrease in MG antibody titers. Finally, group 4 (MG infection and probiotic), 5 (MG infection and Glycyrrhizic acid), and 6 (MG infection and Tylosin) were displayed a significantly moderate MG titer levels between control and infected groups in the end of experiment (Table 1).

Table 1 Comparisons of MG antibody titer among different chicken groups at 10th, 26th, and 35th day of the experimental study.

Group	1st day	10th Day	26th Day	35th Day
G1 (CN1)	1328.9± 124.0	359.75 ±52.1 C	139 ± 34.4 D	74 ± 34.46 D
G2 (MG)	1328.9± 124.0	1281.5± 77.6 A	5578.75±450.6 A	7996 ± 543.63 A

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G3(MG-NDV)	1328.9± 124.0	1347.75±180.1 A	4928.75±454.1 A	7303 ± 319.57 A
G4 (MG-Pro.)	1328.9± 124.0	927.25 ± 48.9 B	1066.75 ± 45.3 C	2770 ± 125.91 C
G5 (MG- GA)	1328.9± 124.0	935.5 ± 43.09 B	1217.5 ± 108.4 C	2227.25±141.41 C
G6 (MG-TYLO)	1328.9± 124.0	1272 ± 77.8 A	2600 ± 174.3 B	5244.75±532.97 B
LSD		336	927	2058
The differences in uppercase letters vertically indicates on significant differences at P≤0.05.				

This study estimated MG antibodies titers, group of MG infection and group of MG infection with ND vaccine were the most groups induced immune responses and antibodies titers against MG infection in the experiment, and maintaining these titers levels across all three times of experiment. Whereas, groups treated with probiotic, Glycyrrhizic acid and Tylosin were showed with moderate immune response and MG antibodies titers between control and infected groups, this result indicated that this treatment enhancing resistance the MG infection through role the probiotic in the decrease the lung injury by reducing MG colonization and pro-inflammatory cytokines and improving the role of microbiota in the MG infection **(23)**. Bacillus subtilis KC1 prevents *Mycoplasma gallisepticum*-induced lung injury by enhancing intestinal *Bifidobacterium animalis* and regulating indole metabolism in chickens. Glycyrrhizic acid have pharmacological properties as hepatoprotective, anti-asthmatic, antioxidative, immunoregulatory effects, antimicrobial and anti-inflammatory **(24)**. Glycyrrhizic acid is used to treat many respiratory diseases, GA may be increased the resistance of MG infection and inhibited inflammatory factors, GA showed that can treat the lung damage in MG infection **(25)**. These references about roles the probiotic and

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Glycyrrhizic acid were explained the moderate MG antibody titers in treatment group because probiotic and Glycyrrhizic acid main role is the preventing the MG infection and decline the damage which caused by MG infection.

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