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RESEARCH ARTICLE

Mycoplasma gallisepticum Infection and Efficacy of Inactivated Poultry Viral Vaccines

Marwa Fathy[1], Mounir M. El-Safty[2], Howaida I. Abd-Alla[3*], Mohamed A. Ibrahim[3, 4], Mohammad K. Ashfaq[4], Jakeen El-Jakee[5]

1. *Bacterial Sterility Department, Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt.*
2. *Quality Control of SPF Eggs Department, Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt.*
3. *Chemistry of Natural Compounds Department, National Research Centre, 12622, Dokki-Giza, Egypt.*
4. *National Centre for Natural Products Research, School of Pharmacy, The University of Mississippi, University, MS, 38677, USA.*
5. *Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.*

***Corresponding Author Email:** *Howaida I. Abd-Alla*

Abstract

Objective: This study was designed to evaluate the effect of *Mycoplasma gallisepticum* (MG) infection on the antibody titers of viral vaccines in fourteen-day-old specific pathogen-free (SPF) chicks. Methods: Five types of inactivated poultry viral vaccines-Newcastle disease viral vaccine (NDVV), avian influenza viral vaccine (AIVV), infectious bronchitis viral vaccine (IBVV), infectious bursal disease viral vaccine (IBDVV) and Reo-Virus Vaccine were used to vaccinate five hundred chicks. The chicks were divided into twenty groups at the age of 14 days; five groups were infected with MG. At day twenty-one, all the groups were vaccinated (four groups for each vaccine one of them previously infected by MG and one simultaneously infected by MG). At day twenty-seven, five vaccinated non-infected groups were infected. Results: All pre-infected groups showed highest antibody titers against NDV, AIV, IBV, IBDV, and Reo V (7.4 log2, 7.1 log2, 20EU, 20EU, and 17EU, respectively) 28 days post-vaccination. The lowest titer appeared in non-infected vaccinated groups with NDV, AIV, IBV, IBDV, and Reo V (6.5 log2, 6.5 log2, 12EU, 14EU, and 11EU, respectively). Conclusion: This study highlighted the outcome of poultry vaccination with inactivated virus vaccines by using serological immune response especially in the presence or absence of *Mycoplasma* infection of chicks.

Keywords: *Mycoplasma gallisepticum, Poultry, Vaccine evaluation, SPF chicks, Serological immune response.*

Introduction

Poultry is widely used as a source of animal protein. It plays a very important role for mankind through food supply, economic uplift, as a source of raw materials to related industries and as a bird model for research [1, 2]. Its meat is popular among Egyptian consumers across all income categories, because of its relatively low cost compared to red meat and fish. The most damaging effects on the profitability of commercial operations are infectious diseases. Among these infections, respiratory viral diseases constitute one of the major health problems as they spread quickly among flocks and can reach 100% morbidity in less than a week [1-3]. *Mycoplasma* infection affects all ages of chicken but young birds are the most susceptible. Interaction of *Mycoplasma gallisepticum* (MG) and viral infections, such as Newcastle disease (NDV), avian influenza (AIV), infectious bronchitis (IBV), and infectious bursal disease (IBDV), exacerbate respiratory diseases and known to inflict heavy losses and thus merit high economic

importance [2, 3]. Moreover, avian Reo V is widespread and has been found in healthy poultry birds [4]. These are known to have an immunosuppressive activity that could allow other organisms (bacteria, parasites, fungi, and viruses) to co-infect the birds [5].

Concurrent infections with Reo V present severe disease condition that hampers proper diagnosis and prevention of the diseases. In view of the impact of these diseases on the global poultry economy, they are listed as notifiable diseases by the Animal Health World Organization-OIE. Due to high pathogenicity of viral diseases such as velogenic and mesogenic NDV, IBV, IBDV, and Reo V, control of these diseases requires well thought out strategies. Beside good management and enhanced biosecurity measures, the control of these diseases is based upon rigorous vaccination of birds with live attenuated and/or inactivated vaccines.

Generally, vaccines induce high and uniform levels of protection after administration of a live vaccine; however, vaccines must meet numerous strict qualities, efficacy, and safety criteria. The criteria for veterinary vaccines are well regulated by several bodies and testing requirements that have been established for potency, efficacy, safety, and purity [6]. The use of these vaccines in poultry is intended to avoid or minimize the emergence of clinical disease at farm level, thus increasing production [7]. Live-virus vaccines are commonly produced by using chicken embryonated eggs [3, 8]. Species of *Mycoplasma* (*M. synoviae MS*), *MG*, and *M. pullorum*) have been isolated from infected embryonated chicken eggs [9, 10].

Mixed infections involving *Mycoplasma* respiratory viruses are under investigation and the outcome of such co-infections is speculated to depend on several factors associated with the host and the organism [11]. The adverse effect of MG contaminated eggs for cultivation of viral vaccines has underscored the importance of specific pathogen free eggs for the production of virus vaccines [12]. The protective efficacy of a vaccine depends on its capability to induce a vigorous and long-lasting immune response.

Chickens are considered the most widely studied avian species showing some differences in immunological response [13]. Therefore, a number of factors such as vaccine doses, routes of administration, protocols, and infection with some avian pathogens such as MG(before, with or post vaccination)may have a significant impact on the safety and efficacy of the vaccines [7]. The viral/bacterial interaction in the same host occurs in nature. In some instances, it shows a beneficial outcome while in others, the outcome is devastating. The effect of these microbes on the immune system is a key factor in determining the cause of the final outcome of such interactions.

Experimentally, one agent can interfere with other, or with the host's defense mechanism [14, 15]. Co-infections cause severe pathological symptoms compared to single infections [16, 17], for example, co-infection of *Mycoplasma* and other viruses may lead to severe pneumonia. Therefore, conflicting results have been reported when comparing a single viral infection to multiple pathogens [18].

Two species of *Mycoplasma*; MG, and MS are pathogenic for chickens and turkeys and seriously affect the global poultry industry [19]. Occasional existence of co-infections of *Acheloplasma* and *Mycoplasma* has been confirmed [12, 20], this incidental finding may have occurred due to immunosuppressant effect of *Mycoplasma* on the host. It is reported that *Mycoplasma* may suppress or stimulate the immune system by affecting the effectors cytokines [19, 21] that interfere with the host response to infectious agents [22, 23]. This study was initiated to study the interaction between MG and selected inactivated viral poultry vaccines (NDVV, AIVV, IBVV, IBDVV, and Reo V) and to evaluate the effect of MG infection on the humoral immune response of chickens against these vaccinations in fourteen-day-old specific pathogen-free (SPF) chicks.

Materials and Methods

Specific Pathogen–free (SPF) Chicks

Fertile SPF chicks (n=500) of fourteen day old were obtained from the National Project for Production of Specific Pathogen Free Eggs (Nile SPF), Koom Oshiem, Elfayoum governorate, Egypt. All 500 were vaccinated at the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt.

MG strain and anti-MG serum were obtained from the *Mycoplasma* Department, Animal Health Research Institute, Dokki-Giza, Egypt. MG strain was propagated as

previously described [24]. Viable counts of MG colony forming units were determined. The inoculums contained10^6cfu of MG strain. The volume of inoculums (100 uL) was given to each chick *via* the intra-nasal route. MG colored antigen was purchased from Intervet International B.v., Boxmeer, and Holland (Summit, NJ, 07901) for the serum plate agglutination testing.

Viruses and Vaccines

NDV strain (genotype 7 accession No.KM288609), AIV (106^{th} embryo lethal dose $(ELD)_{50}$ /bird H5N1 accession No. AFI44355), IBV (viral titer $10^{3.5}$ /mL), IBDV (viral titer $10^{2.0}$ /mL), and Reo virus (titer $10^{3.5}$ /mL) were obtained from viral strain bank of CLEVB. These viruses were used in the challenge tests for vaccinated chicks. Five types of inactivated viral poultry vaccines NDVV, AIVV, IBVV, IBDVV, and ReoVV were obtained from CLEVB. The vaccines were kept at (4- 8 ºC) till use.

Experimental Design

A total number of 500 SPF chicks were divided into 20 groups (25 chicks /group) and placed into separate safety isolators with high biosecurity. Vaccination of groups with different viral vaccines was given on day 21.

To ensure that chicks were experimentally infected with *MG* infection, they were inoculated via two routes intranasal and subcutaneous. A volume of 250 uL of the infectious material suspension containing 10^6cfu of *MG* was instilled in the nasal sinuses as well as injected subcutaneously. The treatment regimens of each group are given in Table 1.

Table 1: Experimental groups vaccinated with different virus vaccines, infected with *Mycoplasma gallisepticum* at different days and subsequently challenged with respective viruses

Group n=5/group	Vaccination (day 21)	MG infection	Virus Challenge (day 28 post vaccination)
1	NDV	On day 16 (IN and SC route)	NDV Genotype 7 (IM)
2	NDV	On day 21 (IN and SC route	NDV Genotype 7 (IM)
3	NDV	On day 27 (IN and SC route)	NDV Genotype 7 (IM)
4	NDV	No MG infection	NDV Genotype 7 (IM)
5	AIV	On day 16 (IN and SC route)	AIV (IM)
6	AIV	On day21 (IN and SC route)	AIV (IM)
7	AIV	On day 27 (IN and SC route)	AIV (IM)
8	AIV	No MG infection	AIV (IM)
9	IBV	On day 16 (IN and SC route)	IBV (M41) (IN)
10	IBV	On day21 (IN and SC route)	IBV (M41) (IN)
11	IBV	On day 27 (IN and SC route)	IBV (M41) (IN)
12	IBV	No MG infection	IBV (M41) (IN)
13	IBDV	On day 16 (IN and SC route)	IBVD (52/70) (conjunctiva)
14	IBDV	On day21 (IN and SC route)	IBVD (52/70) (conjunctiva)
15	IBDV	On day 27 (IN and SC route)	IBVD (52/70) (conjunctiva)
16	IBDV	No MG infection	IBVD (52/70) (conjunctiva)
17	ReoV	On day 16 (IN and SC route)	Reo V (S-1133) (foot pad)
18	ReoV	On day 21 (IN and SC route)	Reo V (S-1133) (foot pad)
19	ReoV	On day 27 (IN and SC route)	Reo V (S-1133) (footpad)
20	ReoV	No MG infection	Reo V (S-1133) (foot pad)

NDV: Newcastle disease virus, AIV: Avian influenza virus, IBV: Infectious bronchitis virus, IBDV: Infectious bursal disease virus, Reo V: Reo virus, IN: intranasal, SC: subcutaneous, IM: intramuscular.

Compliance with Ethics Requirements

The chicks 'care and experimental protocols were in compliance with guidelines of ethical standards released by Cairo University Policy on animal care and use. All efforts were made to ensure ethical and humane treatment of the chicks.

Confirmation of MG Infection

To confirm MG infection of experimentally infected chicken, swabs were taken from the eyes, nose, and pharynges of the infected chickens (pre-, simultaneous, and post-infected groups). Swabs were dipped into pleuropneumonia-like-organisms (PPLO) broth to enhance isolation of *Mycoplasma* and incubated at 37 ºC in a CO_2 incubator (10% CO_2) for 48-72 hours or until the color of the broth changed. Then a loopful of the cultured broth was placed on PPLO agar plate [25] and incubated at 37 ºC in a CO_2 incubator for 5-7 days. Plates were examined daily under as stereo-zone microscope for identification of egg fried colonies specific to *Mycoplasma*.

Serological identification by using SPA test to ensure interaction of MG with vaccination with viral vaccines was done on serum samples separated from all groups at 14, 21, and 28 days post vaccination. MG antibody titer was determined according to method described by Zute et al. [26].

The SPA test was designed as follow: 20 µL of stained MG antigen was placed on a clean glass slide or plate. Followed by addition of 20 µL of standard MG antiserum (control positive slide). For negative control, 20 µL of PBS and 20 µL standard MG antiserum was placed on a clean glass slide or plate (control negative slide). Serum sample (20 µL) from each chick was placed on a clean glass slide or plate.

Followed by adding 20 µL of standard MG antigen. The mixture on each slide was spread by using a glass rod over a circular area of approximately 1.5 cm diameter and rotate the slide for 2 minutes. Result was observed within 2 minutes at room temperature. Positive result for tested samples indicated by formation of agglutination which appear as flocculation of the antigen within 2 minutes, any result after 2 minutes considered negative.

Determination of Antibody Titers after Vaccination

Serum antibody titers were determined to assess the potency of each inoculated inactivated viral poultry vaccine in infected and non-infected vaccinated groups by specific tests of each vaccine. The serum samples collected from the groups 1-4 were used for the estimation of antibody titers against NDV by standard ND antigen [4 haemagglutinating (HA) units].

The groups 5-8 were tested to estimate the antibody titers against AIV using haemagglutination inhibition (HI) test by standard AI antigen (4 HA units). HI test was carried out using the technique described by Allan and Gough (1974). Serum samples collected from the groups 9-12 were tested to estimate the antibody titers against IBV.

The groups 13-16 were tested to estimate the antibody titers against IBDV. The groups 17-20 were tested to estimate the antibody titers against Reo using enzyme-linked immunosorbent assay (ELISA) [27].

Virus Challenge

At 28 days post-vaccination the vaccinated groups were challenged (Code of American Federal Regulations National Archives and Records Administration Biological Products 2012) as given in Table 1. Ten birds from the NDVV vaccinated groups and the control group (groups 1-4) were challenged intramuscularly with a local virulent strain of NDV (Genotype 7 accession No. KM288609) containing 10^6 median embryo lethal dose $(ELD)_{50}$/ bird, all challenged birds were observed for six days post challenge with daily recording of the positive cases(nervous manifestations as lateral deviation of head and neck, off food and weight loss) for calculating the protection percentage.

Ten birds from AIVV vaccinated groups and the control groups (groups 5-8) were challenged intramuscularly with a local virulent strain of AIV (Accession No. AFI44355) contains at least $10^{6.0}$ ELD_{50}/ bird; all challenged birds were observed for deaths and clinical signs as congestion and cyanosis of comb, wattle and legs for 10 days. The tracheal swabs were taken for virus isolation and daily recording of the positive cases for calculation of the protection percentage. Ten birds from IBV vaccinated groups and the control groups (groups 9-12) were challenged with a standard virulent strain of IBVV (M41 type).

Each bird received at least $10^{3.5}$ ELD_{50} of challenge IBV intranasal. All challenged birds were observed for 14 days and tracheal swabs were taken for further virus isolation. Ten birds from IBDVV vaccinated groups and (groups 13-16) were challenged with a standard virulent strain of IBDV (52/70). Birds received at least $10^{2.0}ELD_{50}$ of challenge IBDV *via* conjunctival instillation.

According to Eterradossi et al. [15], the challenged chicks were kept for 10days then scarified and examined for any bursal changes (inflammation, hypertrophy, odema, and hyperemia) at necropsy. Ten birds from Reo VV vaccinated groups (groups 17-20) was challenged with a standard virulent strain of Reo V(S-1133). Bird at least $10^{3.5}$ EID_{50} of challenge Reo V virus into the foot pad 0.1 mL/ chick. One-foot pad of each chick was examined for swelling at least 14 days post challenge.

Protection against Challenge

The protection against the virus challenge was calculated as per cent protection using the following equation: Percent protection = (Number of challenged birds with no symptoms)/ (total number of challenged birds) X 100. This is the standard method adopted for evaluation of veterinary biologics of inactivated poultry viral vaccines in Egypt.

Data Analysis

Data was analyzed using One-Way ANOVA and Student's *t*-test with significance threshold at $P \leq 0.05$.

Results

Identification of MG among the Infected Groups

All swabs (eye, nasal, and pharyngeal swabs) collected from the MG infected groups (pre-, simultaneous- and post-infection) grew fried egg colonies, which were visible under stereo microscope. Also, all serum samples collected from the MG infected groups had clear agglutination using SPA test, while the non-MG infected groups were negative for agglutinations.

Immune Response to Viral Vaccines among the Vaccinated and MG Infected Groups

Significant rise in antibody titers were observed on day 14, 21, and 28 post vaccinations. The highest titer of NDV antibodies by HI test on days 28post vaccination was in the *MG* pre-infected group (7.4 log2) while the lowest titers were recorded in the NDV vaccinated non-infected group (6.5 log2). The protection percentage against NDVV revealed that the MG pre- and post-infected, ND vaccinated groups were more protected group (100% each) than simultaneously MG infected and non-infected NDVV vaccinated groups (90% each) Similarly, antibody titers against AIV showed significant increase on day 14, 21, and 28 post vaccination (Table 2).

Table 2: The average of antibody titers against ND and AI inactivated vaccines tested by haemagglutination inhibition (HI) test and protection against virus challenge

Groups	Day of post-vaccination			*t*-Test		Percent protection*
	14th	21th	28th	Sig. (2-tailed)	SD	
G1	3.4 log2	6.1 log2	7.4 log2	0.041	2.040	100
G2	3.0 log2	5.6 log2	6.8 log2	0.045	1.942	90
G3	3.1 log2	5.9 log2	7.0log2	0.044	2.010	100
G4	2.8 log2	5.3 log2	6.5 log2	0.047	1.887	90
G5	3.4 log2	6.1 log2	7.1 log2	0.038	1.913	100
G6	3.2 log2	5.8 log2	6.8 log2	0.039	1.858	90
G7	3.0 log2	5.6 log2	6.6 log2	0.042	1.858	100
G8	2.8 log2	5.3 log2	6.5 log2	0.047	1.887	90

*= The protection % was calculated post-challenge test, Sig. = Significance ($P \leq 0.01$- 0.05), SD: Standard deviation, G1: Group 1 infected with MG then vaccinated with NDVV, G2: Group 2 vaccinated with NDVV and simultaneously infected with MG, G3: Group 3 vaccinated with then infected with MG, G4: Group 4 vaccinated only with NDVV, G5-8: Groups 5 - 8 vaccinated with AI VV as in groups 1 - 4. For calculation of the protection % we use ten birds in the challenge test and record the deaths after challenge test then calculate the protection %

The highest titer of AI antibodies by HI test at 28 days post vaccination was in *MG* pre-infected group 7.1 log2 while the lowest titers were recorded in AI vaccinated non-infected group 6.5 log2. The protection percentage (Table 2) against AIVV indicated the MG pre- and post-infected, AIVV vaccinated groups were more protected group (100 % each) than simultaneously MG infected and non-infected AIVV vaccinated groups (90 % each).

Table 3: Antibody titers against IB, IBD and Reo inactivated vaccines tested by ELISA and protection against virus challenge among the vaccinated groups

Groups	Day of post-vaccination			*t*-Test		Percent Protection***
	14th	21th	28th	Sig. (2-tailed)	SD	
G9	8 EU	18 EU	20 EU	0.053	6.110	100
G10	7 EU	14 EU	17 EU	0.051	5.132	90
G11	6 EU	13 EU	14 EU	0.049	4.359	90
G12	5 EU	11 EU	12 EU	0.051	3.786	90
G13	10 EU	18 EU	20 EU	0.035	5.292	100

G14	8 EU	16 EU	18 EU	0.044	5.292	100
G15	7 EU	15EU	17 EU	0.047	4.932	100
G16	6 EU	13 EU	14 EU	0.049	4.359	90
G17	7 EU	14 EU	17 EU	0.051	5.132	100
G18	6 EU	13 EU	14 EU	0.049	4.358	90
G19	5 EU	11 EU	12 EU	0.051	3.785	90
G20	5 EU	11 EU	11 EU	0.046	3.464	90

*** 6th days post-challenge, EU: ELISA unit, Sig.: Significance (0.01-0.05), SD: Standard deviation, G9-12: Groups 9 – 12 vaccinated with IB VV, G9: Group 9 pre-infected with MG then vaccinated with IBVV, G10: Group 10 vaccinated with IBVV and simultaneously infected with MG, G11: Group 11 vaccinated with IB VV then infected with MG, G12: Group 12 vaccinated only with IB VV G13-16: Groups 13 - 16 vaccinated with IBD VV as in groups 9–12, G17 and 18: Groups 17 and 18 vaccinated with Reo VV as in groups 9–12

As shown in Table 3, there was significant rise in antibody titers after vaccination against the respective viruses. The highest titer of IB antibodies by ELISA test on day 28 post vaccination was in group 9 (20 EU) while the lowest titers were recorded in IB group 12(12 EU). The protection percentage against IBV revealed that group 9 was the most protected group (100%) compared with the other groups. The highest titer of IBD antibodies by ELISA test at 28 days post-vaccination was in the MG pre-infected IBD vaccinated group (20 EU) while the lowest titers were recorded in MG simultaneously infected IBD vaccinated group (14 EU).

The protection percentage against IBDV revealed that the MG non-infected IBD vaccinated group was the lowest protected group (90 %) compared with the other groups. The highest titer of Reo antibodies by ELISA test at 28 days post-vaccination was in MG pre-infected Reo vaccinated group (17 EU) while the lowest titers were recorded in the Reo vaccinated non-infected group (11 EU). The protection percentage against Reo revealed that the MG pre-infected, vaccinated group was the most protected group (100 %) compared with the other groups (Table 3).

Discussion

The protective efficacy of a vaccine depends on its capability to induce vigorous and long-lasting immune response. However, in poultry vaccinations, presence of other microorganisms such as *Mycoplasma* contaminated vaccine or *Mycoplasma* infection of host may produce varying degree of immunological response [7]. Conflicting results have been reported from *Mycoplasma* with multiple viral infections compared to a single infection, where multiple pathogens infections present shown clinical picture with high degree of severity [18].

The purpose of this study was to detect the influence of *Mycoplasma* infection on the efficacy of some inactivated poultry viral vaccines and to evaluate the protection provided by vaccination against viral challenge. MG is a natural respiratory pathogen of the chickens and well recognized as an exacerbating factor in poultry viral respiratory diseases such as ND disease and IB [28, 29]. MG is one of the major agents in multifactorial disease complex. It predisposes birds to action of some vaccine strains as ND or IB, and infectious laryngotracheitis (ILT) viruses [30].

The interaction between pathogens has been observed between many bacterial diseases and the outcome could be either synergistic or antagonistic positive interactions as have been observed in AI-infected birds and MG or MS. The outcome of this phenomenon depends on interaction time (pre-, simultaneous, or super infection), host immune response, biological product, and/or other environmental factors [31].

Hopkins and Yoder [32] suggested that chicken-passaged mild IB virus vaccine in combination with MS markedly increased the incidence of air sacculitis compared to non-passaged virus vaccine so that chicks vaccinated with IB combined with *MS* vaccine have higher antibody (AB) titre and more protection against the IBV disease alone.

The conclusion drawn from that study was that IB infection may precipitate latent *Mycoplasma* infection and emphasized the importance of using *Mycoplasma* free chickens for the evaluation of the efficacy of viral vaccines. The present work was planned to study the interaction effect of prior, simultaneously, and post *Mycoplasma* infection on the immune response against ND, AI, IB, IBD, and Reo viral vaccines in

different chicken groups comparing to non-infected vaccinated groups.

The results supported the observation of Bolha et al. [33], when mildly virulent virus strains infected birds that had already been infected with *Mycoplasma*, the interactions between the host, *Mycoplasma* cells, and virus could result in effects that differ from those of single pathogen infection. Thus, severe inflammatory reactions and synergistic pathogen interactions can occur after vaccinations with live virus vaccines.

In the recent years, co-infections were slightly increased, and could be found in severe cases [34], such as bacterial co-infections with influenza were associated with more severe illness and worse outcomes [35]. Similarly, in 2004, Landman and Feberwee recorded the increase of the severity of the clinical respiratory symptoms associated with *M. synoviae* by co-infection with various respiratory viruses such as ND and IB [36]. In our study MG strain may have interfered with AB titers of the inactivated NDV, AIV, IBV, IBDV, and Reo vaccines. Mycoplasmas may affect the cell-mediated immune system by inducing either suppression (in chronic phase of infection) or stimulation (in acute phase of infection) of B and T lymphocytes [21] as observed in the present experiment where MG elevated the antibody titers against NDV, AIV, IBV, IBDV, and Reo vaccines during the acute phase of *Mycoplasma* infection. Results in the present study indicated that the highest titer of ND antibodies by HI test at the 28 days post vaccination in *Mycoplasma* pre-infected group was 7.4 Log2 compared with the lowest titer recorded in ND vaccinated non-MG infected group (6.5 logs 2).

These results are in contrast to those reported by Silva et al., where *MS* infection followed by ND vaccination seven days later, yielded higher and longer lasting serologic responses to ND vaccine in the non-*MS* infected chicks compared to those infected and vaccinated chicks [1]. Different effects of MG and MS on the antibody response to ND vaccination remain to be elucidated.

Percentage protection in groups vaccinated with NDV revealed 100% protection. This is based on appearance of the clinical signs of the chickens that were infected with MG pre- and post-vaccination with ND vaccine and then challenged with NDV. The group that was simultaneously infected with MG and vaccinated with ND vaccine showed 90% protection against NDV virus challenge as some chickens in this group showed signs of the disease after challenge.

In 2004, Landman and Feberwee recorded the increase of the severity of the clinical respiratory symptoms associated with *M. synoviae* by co-infection with various respiratory viruses such as ND and IB [36]. Stipkovits et al. concluded that the birds co-infected with MG and low pathogenic avian influenza strain A/ mallard/ Hungary/ 19616/07 (H3N8) showed clinical signs and pathologic lesions and reduction in weight gain [30]. Similar observations were in the present study. Subtain et al. [37] recorded that MG caused more severe clinical signs and increased antibody titer when chickens were co-infected with AI than the other factors in AI sub type H9N2 infection in chickens. The reason for the enhanced pathogenicity could be the release of proteases enzymes through the replication of bacteria such as MG.

Clearly, the highest titer of AI antibodies by HI test at 28 days post-vaccination in the *Mycoplasma* pre-infected group was 7.1 log2 while the lowest titers were recorded in the AI vaccinated non-infected group. These results supported what has been reported by Thacker et al. [38], in which an experimental respiratory model was used to investigate the interaction between *M. hyopneumoniae* and swine influenza virus.

Also, in our study, the results demonstrated that a protection against AIV challenge in the *Mycoplasma* pre- and post-infected AI vaccinated groups was 100% while in the *Mycoplasma* infected and non-infected AI vaccinated groups was 90%. Subtain et al. [37] challenged different groups of chickens with MG and H9 virus to observe the role of MG infection in exacerbating pathogenicity of H9 virus. In the presented study, *Mycoplasma* pre-infected group showed the highest titer of IB antibodies by ELISA test (20 EU) at the 28 days post vaccination while the lowest titers were recorded in IB vaccinated non-MG infected group.

Also, MG pre-infected, IBV vaccinated group was the most protected group (100%) compared with the other groups. In 1984, Bradbury [28] demonstrated synergism between MG and the viruses of ND and IB

although the outcome of infection was influenced by many factors associated with the host and the organisms. The data shows the highest titer of IBD antibodies by ELISA test

at the 28 days post vaccination in *Mycoplasma* pre-infected IBD vaccinated group (20 EU) while the lowest titer recorded in *Mycoplasma* simultaneously infected IBD vaccinated group. This is in contrast to that reported by Yagihashi et al. [39] who studied the effect of previous infection of 8-week old chicken with IBDV and their susceptibility to MG and MS, where antibody titers against MS and IBDV were detected by using HI and immune-diffusion test [39]. The results indicated that concomitant infection of MG acted synergistically with MS and that previous exposure to IBDV increases the susceptibility to MS infection.

Results of groups vaccinated with Reo indicated that the highest titer of Reo antibodies by ELISA test at the 28 days post vaccination in *Mycoplasma* pre-infected Reo vaccinated group is (17EU) while the lowest titers were recorded in Reo vaccinated non-infected group.

Al-Afaleq et al. [40] reported that the Reo virus neutralizing antibodies were detected at 3 weeks and persisted until the end of the experiment at 15 weeks in the dually-infected group (infected with reo virus together with the MS). Reck et al. [41] reported the histopathological changes caused by mixed infection with MS and avian *ortho*-Reo virus in broilers. Mixed infection between *MS* and avian *ortho*-Reo virus suggested the presence of synergistic relationship. In the present study, the protection percentage against Reo virus suggested that the *Mycoplasma* pre-infected Reo vaccinated group was the most protected group (100%) compared to the other vaccinated groups.

Bradbury and Garuti [11] as well as Al-Afaleq et al. [40] indicated that Reo viruses have been shown to interact with MS and these strongly support our findings. With that stated our studies ascertains the interaction between *Mycoplasma* and viral vaccination and highlights the efficacy of selected inactivated poultry viral vaccines as beneficial tool to improve the vaccination programs in poultry farms.

Conclusion

This study highlighted the outcome of poultry vaccination with inactivated virus vaccines by using serological immune response, especially in the presence or absence of *Mycoplasma* infection of chicks. All pre-infected groups showed significant antibody titers against ND, AI, IB, IBD, and Reo (7.4 log2, 7.1 log2, 20 EU, 20 EU, and 17 EU, respectively) 28 days post-vaccination. The purpose of the current study is to explain that there is a significant and clear interference effect of MG in the serological immune response to inactivated viral vaccines of poultry (AI, ND, IB, IBD and Reo as a model). The results of this has raised concern to explore the possible on the causes of false results in the evaluation process of some viral poultry vaccines, especially when chickens were infected previously with *Mycoplasma*.

It is hoped that this present study will help the researchers to avoid evaluation of inactivated viral poultry vaccines when chicks are previously been exposed to Mycoplasma infection. The study underscores the importance of the using SPF chickens in evaluation process of the poultry vaccines to avoid the interaction effects of two or more agents (as MG and other viral vaccines) so it can obtain a considerable and reliable evaluation results of many vaccines.

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