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Study the Feedback of Mycoplasma Gallisepticum Infection on the Efficacy of Inactivated Viral Vaccines of Poultry

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Abstract

The present study monitored the effect of Mycoplasma gallisepticum infection on the humeral immune response of chickens against the different inactivated bivalent vaccines (ND, AI), (ND, IB), (ND, IBD) and monovalent (Reo Vaccine). These inactivated vaccines were used to inoculate three hundred 3-week-old specific pathogen-free (SPF) chicks by S/C route. They were grouped into nine groups; Groups (1, 3, 5 and 7) pre-infected with MG one week before vaccination and Groups (2,4, 6 and 8)vaccinated and non-infected. Group (9) was kept as control. In this study field infection of Mycoplasma gallisepticum was designed by inoculation of 250uL of the infectious material suspension containing 10^6cfu of MG was instilled in the nasal sinuses as well as injected subcutaneously one week before vaccination. The immune response was estimated and evaluated by using HI, ELISA and challenge tests. The bacterial stress of M. gallisepticum was recorded by the lesion scores. The results of HI and ELISA tests for the pre-infected groups showed highest antibody titers against (ND, AI), (ND,IB), (ND,IBD), and Reo vaccines which were significantly(at $P \geq 0.05$) higher (7.7 $\log_2$, 7.8 $\log_2$, 7.9 $\log_2$, *respectively)for ND,(7.1 log_2)for AI antigen, andS/P ratio showed non significant increase (1.7, 1.7, 0.3) for IBV, IBDV, and Reovirus antigens respectively28 days post-vaccination. The lowest titer appeared in non-infected vaccinated groups (G2, G4 and G6) (6.5 log_2, 6.7 log_2, 7.1 log_2, respectively) for ND, (6.5 log_2)for AI antigen, and S/P ratio was (0.6, 0.8, 0.28) for IBV, IBDV, and Reovirus antigens respectively. This study used serological immune responses to emphasize the results of chicken vaccination with inactivated viral vaccines, particularly in the presence or absence of Mycoplasma infection in chicks. Indeed, there is evidence that vaccinated chicken flocks pre-infected with MG can introduce inaccurate results compared to non infected vaccinated flocks. For the previous conclusion the evaluation of any inactivated viral vaccine must be performed in free Mycoplasma chickens, (SPF chickens as an example) in order to obtain accurate evaluation results.*

Keywords: *Avian influenza, IB, IBD, Inactivated Vaccine, Mycoplasma gallisepticum, HI, SPF chicks.*

Introduction

Poultry represents one of the most important sources of protein for egyptian consumers across all categories of society,as it has low cost compared to fish and red meat. The most damaging effects on the revenue of commercial and industrial operation are infectious diseases.Among these infectious diseases, respiratory viral diseases are considered one of the major health problems as they have high spread rate among poultry flocks and can reach a morbidity of 100% in less than a week (**Sid et al.,2015)**. Chickens are considered the most widely studied avian species which show some variations in immune response (**Abd-Alla et al., 2017)**. The safety and effectiveness of the immunizations may be significantly conducted by a number of variables, including vaccine doses, administration methods, protocols and infection with specific avian infections such as MG (**Marangon and Busani, 2006)**. In nature, there is a viral/bacterial interaction on the same host. It demonstrates advantageous outcomes in some cases and disastrous outcomes in others. The impact of these bacteria on the immune system is a crucial consideration in figuring out what ultimately led to these interactions. Mycoplasmamay suppress or stimulate the immune system by affecting theeffectorscytokines(**Volokhov et al.,2011)** that interferewith the host response to infectious agents(**Chen et al.,2016)**.

Mycoplasma, a large group of prokaryotes thought to have evoluted from Gram-positive bacteria by degenerative evolutionmainly capable of causing a variety of human and animal infectionsas mentioned by**Lianmei et al. (2019).** Mycoplasma gallisepticum (MG) is a contagious respiratory pathogenamong 22 serotypes (**Yilmaz et al., 2011)** that affects poultry and causeinfectious sinusitis in turkeys**(Kanci et al., 2018)**.It is a significant avian pathogen that causes the chronic respiratory disorders that afflict chicken and turkeys and cause significant economic losses for the poultry sector**(Ishfaq et al., 2020)**.The disease reduces egg production and raises embryo mortality in breeders and layers. In layers, production losses of among ten and twenty percent have been documented. All species of chicken and turkey are susceptible to these illnesses, however birds of young age are highly vulnerable than adult birds (**Zulfekar et al.,2015**).Moreover, high mortality is high, carcassare discarded andfeed conversion rate is low(**Mukhtar et al., 2012).**

Mycoplasmosis is transmitted vertically by broiler breeders via eggs **(Xue et al., 2017)** or horizontally, by direct contact between diseased and carriers **(Marois et al., 2000)**. Moreover,Mycoplasmacan remain in the flock constantly as forms **(Nascimiento et al., 2005)**. Infected birds become asymptomatic carriers and immunocompromised in which MG escapes from immunity and multiplies within macrophage **(Gondal et al., 2013).** It transfers through ovaries to next generation (**Feizi et al., 2013).**

Vaccination used to control infection in countries of significant commercial industries of poultry **(Zhu et al., 2018)**, but the success of these control programs depends on the accurate and timely diagnosis of infected flocks**(Gharibi et al., 2018)**.An adjuvant is preferable to increase both cellular and humeral immunities not onlyan effective vaccine(**Marwa et al., 2021)**.Efforts toprevent the losses in commercialflocks particularly inlayer and breeders including bacterin-based vaccines,killed vaccines, and live vaccines havebeen successful up to some extent in reducing theseverity of respiratory diseases, maintaining constantegg production, controlling excess vaccine reactions,and reducing horizontal and vertical transmission**(Butcher, 2002).**

Several bacterial and viral pathogens, both individually and in combination, have the ability to make poultry susceptible to illness.**(Samy and Naguib, 2018)**, therefore The combination of bacterial and viral vaccines that contain multiple antigens has many advantages for producers because it lowers production costs; for managers because it frees up time and effort and facilitates the immunization schedule; and for animals because it reduces the stress of receiving multiple vaccinations.(**Orabiet al.,2017)**.

There is a role of Mycoplasma gallisepticum infection in accelerating the replication of H9 virus and pathogenicity exacerbationin experimental conditions.The possible reason for the enhanced pathogenicity could be the release of proteases enzymes by the replication of bacteria such as Mycoplasma gallisepticum. These enzymes might recognize a monobasic cleavage signal at HA of influenza virus which plays an important role in the pathogenicity of the virus(**Subtain et al.,2016)**.InteractionofMycoplasma gallisepticum(MG)and viral infections, such as Newcastle disease (NDV), avian influenza (AIV), infectious bronchitis (IBV) and infectious bursaldisease (IBDV),exacerbate respiratory diseases and known to inflict heavy losses and thus merithigh economic importance (**Sid et al.,2015)**.The interactions with(IBV) or (NDV) which have long been known to result in a synergistic effect with M. gallisepticum.These interactions are obtained by using vaccine strains of(IBV) or(NDV) or An effective vaccine(**Kleven,1998)**.Conflicting results have been reported from Mycoplasma with multiple viral infectionscomparedtoa single infection,wheremultiple pathogens infections have showed more severe clinical presentation**(Sly and Jones,2011)**.Mildly virulent virus strains infect birds that had already been infected withMycoplasma, the interactions between the host,Mycoplasmacells 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(**Bolha et al., 2013)**.

Wei Zhang et al. (2020)stated that eradication of the disease in poultry breeder flocks asMycoplasma gallisepticum (MG) causes immune response dysregulation in the lungs and air ways of poultry(**Rabia et al.,2021).** The disease is therefore controlled throughkilled MG **(Olanrewaju et al., 2011),** live vaccines,and recombinant viral vaccines is still a majorconcern **(Ishfaq et al., 2020).**

In Egypt there is a lack of information about the immune reaction to mixed MG and E.coli infections in chickens vaccinated with NDV vaccine. These knowledge gaps encourage the need for more studies to demonstrate the adverse effects of those infections on the immune system at the time of vaccination (**Naglaaetal.,2019**).

Material and Methods

Chickens:

Three hundred, 3-week-old specific pathogen-free(SPF) chicks were obtained from Koom-Osheim Farm in the Fayoum Governorate, Egypt. The chicks were fed add libitumwithout anticoccidial or antibacterial ingredients in their feed.

Mycoplasma gallisepticumstrain:

Local strain isolated by Animal Health Research Institute, Mycoplasma Department, Dokki, Giza, Egypt and propagated as described by**Rodwelland Whitcomb(1983).**

Viral strains used for challenge:

The CLEVB Viral Strain Bank provided the NDV strain 10^6 median embryo lethal dose(ELD)$_{50}$ (genotype 7 accession number KM288609) and AIV (10^6 embryo lethal dose (ELD)$_{50}$/ bird (H5N1 accession number AFI44355),IBV (viral titer $10^{3.5}$/ml), IBDV (viral titer $10^{2.0}$/ml), andReo virus(titer $10^{3.5}$ /ml). Vaccinated chicks were underwent challenge testing using these viruses.

Inactivated viral vaccines:

Three types of inactivated viral bivalent poultryvaccines (ND, AI), (ND, IB), (ND, IBD) andone monovalentinactivated viral vaccine (REO Vaccine)were obtained from CLEVB and were kept at (4 - 8 ºC) till use.

Experimental Design:

A total number of 300 SPF chicks were divided into 8 groups(30chicks/group) and group (9) is the control group(60 chicks) (Table 1). All groups were placed into separate safety isolators with high biosecuritymeasures.Vaccination of groups (from Group 1 to Group 8) with different viral vaccines was given on day 21 shown in Table (1). To ensure that chicks of Groups (1, 3, 5 and 7) were experimentally infected withMGinfection, they were inoculatedvia intranasal route. Group (9) kept as control group for challenge test.

Confirmation of MG Infection

Seven days before immunization with the viral vaccines, chickens of pre MG infected groups (G1, G3, G5 and G7) wereinfectedwith M. gallisepticum strain intra-nasally and ensured the infection via inoculation of M. gallisepticumsubcutaneously.A volume of 250uL of the infectious material suspension containing 10^6cfu of MG was instilled in the nasal sinuses as well as injected subcutaneously, Swabs were obtained directly before vaccination from the eyes, noses and pharynxes of the experimentally infected chickens to confirm MG infection (pre-infected groups). To increase the isolation of Mycoplasma, swabs were placed in pleura-pneumonia-like organisms (PPLO) broth and then incubated at 37°C in a CO_2 incubator (10% CO_2) for 48–72 hours or until the broth's colour changed. Apart of the cultured broth was then spread onto a PPLO agar plate, where it was incubated for 5-7 days at 37^0 C in a CO_2 incubator. Every day, plates were inspected with a stereo-zone microscope to look for egg-fried colonies that were specific to Mycoplasma (**Razin, 1983)**.

Serum samples isolated from all groups on days14, 21 and 28 after immunization with different inactivated viral vaccines were subjected to serum plate agglutination (SPA)test for serological identification to confirm the interaction of MG with viral vaccinations. This test was conducted to determine the existence of MG antibody as described by **Zute and Valdovska(2015)**.

The SPA test was made in the following way: 20µl of stained MG antigen was placed by unichannel on a spotless glass slide or plate followed by addition of 20µl from standard MG antiserum (control positive slide).20µlof PBS and 20µlstandard MG antiserum was placed by unichannel on a clean plate (control negative slide).20µl from serum sample was placed by unichannel on a clean plate followed by adding 20µlof standard

MG antigen from each samble.The mixture on each slide was spread by using a glass rod over a circular area of approximately 1.5 cm diameterand rotate the slide for 2 minutes.Result was observed within two minutes at room temperature. Result after 2 minutes is considered negative. Agglutination formation, which exhibits as antigen flocculation, shows a positive result for tested samples.

Challenge test:

Executed according to the steps described by **OIE(2019).**

Protection % against challenge test:

After 4 weeks from vaccination (WPV), 10 birds from groups (G1, G2, G3, G4, G5 and G6) were challenged intra-nasally (IN) with NDV strain 10^6 median embryo lethal dose (ELD_{50}) (genotype 7 accession number KM288609) and observed for ten days post challenge with daily recording of the positive cases (nervousmanifestations as deviation of head and neck laterally,off-food and weight loss)for calculating the protection percentage as described in Table (1).

At the same time 4 WPV, another 10 birds from groups (G1 and G2)were challenged intra-nasally (IN) with AIV (10^6 embryo lethal dose (ELD_{50})/ bird H5N1 accession number AFI44355)and observed for ten days post challenge with daily recording of the positive cases; the comb and wattle of sick birds showed cyanosis, and they also had echymosis on their shanks, facial edema, greenish diarrhea and nervous manifestations such torticollis and tremors (Table 1).

Ten birds were challenged intra-nasally (IN) 4 WPV with IBV (viral titer $10^{3.5}$/ml)in groups (3 and 4), and via conjunctivawith IBDV (viral titer $10^{2.0}$/ml) in groups (5 and 6), as well as withReo virus(titer $10^{3.5}$ /ml) in groups (7 and 8),Reo virus inoculation into the foot pad 0.1 ml/chick. One-foot pad of each chick was checked for swelling at least 14 days after the challenge (Table 1). Ten birds total for every antigen vaccination type. For 10 days following the challenge (DPC), all hens were monitored and checked daily in order to record mortalities for each group and report any clinical signs.

Table (1): Different vaccinated infected and non infected groups representing different challenge viruses

Groups	**Vaccine type Ag**	**Mycoplasma Gallisepticum (MG) infection**	**Challenge trial (4 weeks post vaccination) (Route)**
Group 1	ND	Infected	NDV Genotype 7 (I.N)
	AI	Infected	AIV (I.N)
Group 2	ND	Not-Infected	NDV Genotype 7 (I.N)
	AI	Not-Infected	AIV (I.N)
Group 3	ND	Infected	NDV Genotype 7(I.N)
	IB	Infected	IBV (M41) (I.N)
Group 4	ND	Not-Infected	NDV Genotype 7(I.N)
	IB	Not-Infected	IBV (M41) (I.N)
Group 5	ND	Infected	NDV Genotype 7(I.N)
	IBD	Infected	IBDV (52/70) (conjunctiva)
Group 6	ND	Not-Infected	NDV Genotype 7(I.N)
	IBD	Not-Infected	IBDV (52/70) (conjunctiva)
Group 7	REO	Infected	Reo V (S-1133) (foot pad)

Group 8	REO	Not-Infected	Reo V (S-1133) (foot pad)
Group 9	Control	Not-Infected, Not-vaccinated	______

Newcastle disease (ND), Newcastle disease virus (NDV),Avian Influenza (AIV), Avian Influenza virus (AI), Infectious Bronchitis (IB), Infectious Bronchitis Virus (IBV),Infectious Bursal Disease (IBD), Infectious Bursal Disease Virus (IBDV),and Reo. I.N=intranasal

Equation for protection%:

The following equation was used to determine the percentage of protection: Protection Percent = (Number of challengedbirds with no symptoms)/ (total number of challenged birds) X100. This is the standard method adopted for evaluation of veterinary biologics of inactivated poultry viral vaccines in Egypt.

Serological testes for detection of humeral antibody response:

a- Haemagglutination inhibition (HI) test for ND and AI:

It was conductedin accordance to **(OIE 2019).**

b. ELISA kits:

These kits were used for detection of IB, IBD and REO antibodies following the manufacturer's instructions.

Serological monitoring of antibodies:

Samples of blood were taken from jugular vein and kept in a slope position at 37^{o}C for one hour then at 4^{o}C overnight. Sera were then separated by centrifugation at 3000 rpm for 10 minutes and stored at -20^{o}C. Sera were inactivated at 56^{o}C for 30 minutes before testing. Ten serum samples were collected from each group(1-8) at14th, 21stand 28th days post vaccination (PV) for post vaccination monitoring. Serum samples were tested usinghaemagglutination inhibition test (HI) in groups containing ND and AIantigensgroups (1, 2, 3, 4, 5 and 6) according to the technique described by **OIE (2019)** with standard (4HA units) for ND and AIantigens.While groups(3,4,5,6, 7 and 8)containing IB, IBD, REO antigens the antibody titers were estimated using enzyme-linked (ELISA) test specific for each antigen according tothe manufacturer's instructions.

Statistical analysis

We analyzed the results of the HI tests **(**Table-2) and ELISA tests (Table-3) and compared the parametrical correlations using Student's t-test **Snedecor (1980**). Significance level was at p>0.05

Results

Confirmation of Mycoplasma gallisepticum infection:

All samples swabbed from the MG infected birds (eye, nasal and pharyngeal swabs) grew fried egg colonies on cultured plates, which were visible under a stereo microscope. Additionally, the SPA test revealed obvious agglutinations in all serum samples taken from the MG-infected groups but not in the non-MG-infected groups.

Serological monitoring of antibodies for different vaccinated groups:

On days 14, 21, and 28 (2nd week, 3rd week and 4th week) following immunization, marked significant (at $P \geq 0.05$)increase in antibody titers was seen. The MG pre-infected group had the greatest titer of NDV antibodies by HI test on days 28 post vaccination in groups1, 3, and 5which were (7.7 $\log_2$, 7.8 $\log_2$, 7.9 $\log_2$, respectively), whereas the NDV vaccinated non-infected groups(2, 4 and 6) had the lowest titers (6.5 $\log_2$, 6.7 $\log_2$, 7.1 $\log_2$,respectively). The MG pre-infected ND vaccinated groups (1, 3 and 5) were more protected (100%, 90%, 100% respectively) than the non-infected NDVV vaccinated groups (2, 4 and 6) (90% for each) according to the protection percentage against NDVV as shown in Table(2) , figure (1).

For AIV, on days 14, 21, and 28 (2nd week, 3rd week and 4th week) following immunization, also antibody titers against AIV significantly (at $P \geq 0.05$) increased inthe MG pre-infected and vaccinated group(7.1 $\log_2$) in group(1) compared to non-infected and vaccinated group(2)as shown a titer of (6.5 $\log_2$).The protection % in the MG pre-infected and vaccinated group(G1) was (90%) while in non-infected and vaccinated group(G2) was (80%)as stated in table (2), figure (1).

Table (2): The mean haemagglutination inhibitiontest (HI) antibody titers and protection%against ND and AI inactivated antigens in different bivalent vaccines

Groups		Days post vaccination Virus titer (Log_2)			Protection % (10th day post challenge)
		2nd week	3rd week	4th week	
Group 1	ND	4.6	6.1	7.7*	100%
	AI	5.1	6	7.1*	90%
Group 2	ND	2.6	4.5	6.5	90%
	AI	4.1	5	6.5	80%
Group 3		5.3	7.0	7.8*	90%
Group 4		4.1	6.1	6.7	90%
Group 5		3.7	7.1	7.9*	100%
Group 6		2.7	6.1	7.1	90%
Group 9		0.0	0.0	0.0	0%

G1: Group 1 infected with MG thenafter 1 week vaccinated with bivalent vaccine (ND, AI),

G2: Group 2 vaccinated with bivalent vaccine (ND,AI),

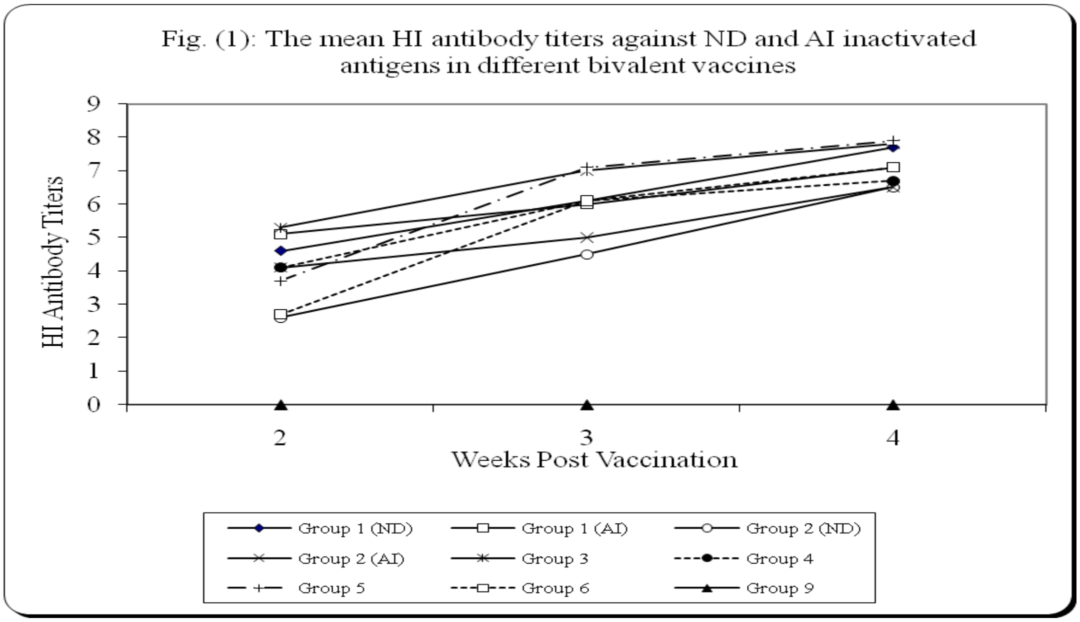
G3: Group 3 infected with MG then vaccinated with bivalent vaccine (ND, IB),

G4: Group 4 vaccinated with bivalent vaccine (ND, IB),

G5: Group 5 infected with MG then vaccinated with bivalent vaccine (ND, IBD),

G6: Group 6 vaccinated with bivalent vaccine (ND, IBD),

The protection % was calculated post-challenge.*Significant at $p>0.05$.



G1: Group 1 infected with MG then after 1 week vaccinated with bivalent vaccine (ND, AI),

G2: Group 2 vaccinated with bivalent vaccine (ND,AI),

G3: Group 3 infected with MG then vaccinated with bivalent vaccine (ND, IB),

G4: Group 4 vaccinated with bivalent vaccine (ND, IB),

G5: Group 5 infected with MG then vaccinated with bivalent vaccine (ND, IBD),

G6: Group 6 vaccinated with bivalent vaccine (ND, IBD),*Significant at p>0.05.

For IB antigen detection by ELISAKit in day 28 post vaccination (4 WPV) in group (3) that representedthe MG pre-infected vaccinated groupS/P ratio of (1.7) and protection % of (90%) that was higher with nonsignificant (at P $\geq$ 0.05) increase than the MG non-infected and vaccinated group (Group 4) (0.6)S/P ratioand protection % of (90%) as detailed in table (3), figure (2). In group (5) the MG pre-infected and vaccinated group containing IBD antigen S/P ratio was (1.7) 4- weeks after vaccination and the protection % was (100%) while in group(6) S/P ratio was (0.8)and the protection % was (90%).In group (7), increase the titer of Reo antibodiesby ELISAtest at 28 dayspost-vaccinationwasinMGpre-infected Reo vaccinated group(0.3) showed nonsignificant (at P $\geq$ 0.05) increase with a protection% (100%), compared to group (8) non-infected vaccinated group with S/P ratio of (0.28)and a protection% (100%)as mentioned in the following Table (3), figure (2).Group (9) represented the control for all groups showed (0%) protection.

Table (3): Antibody S/P ratio against IB, IBD and Reo antigens in different inactivated vaccines tested by ELISA and protection against virus challenge among the vaccinated groups

Groups	Days post vaccination			Protection %
	S/Pratio			(10th day post challenge)
	2nd week	3rd week	4th week	
Group 3	0.217	0.6	1.7	90%

Group 4	0.03	0.4	0.6	90%
Group 5	0.25	0.9	1.7	100%
Group 6	0.12	0.3	0.8	90%
Group 7	0.12	0.23	0.3	100%
Group 8	0.03	0.18	0.28	100%
Group 9	0.001	0.003	0.001	0%

G3: Group 3 infected with MG then vaccinated with bivalent vaccine (ND, IB),

G4: Group 4 vaccinated with bivalent vaccine (ND, IB),

G5: Group 5 infected with MG then vaccinated with bivalent vaccine (ND, IBD),

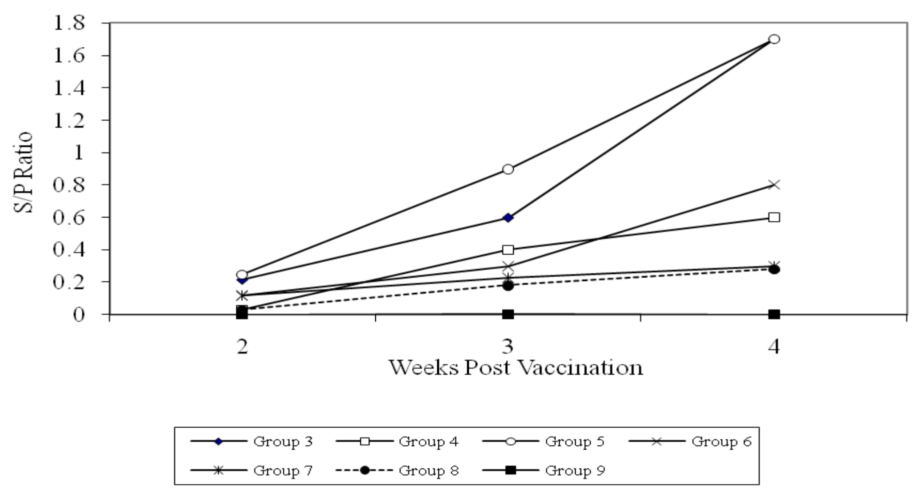
G6: Group 6 vaccinated with bivalent vaccine (ND, IBD),

G7: Group 7 infected with MG then vaccinated with REO vaccine,

G8: Group 8 vaccinated with REO vaccine,

The protection % was calculated post-challenge.*Significant at p>0.05.

Fig. (2): Antibody S/P ratio against IB, IBD and Reo antigens in different inactivated vaccines tested by ELISA in the vaccinated groups



G3: Group 3 infected with MG then vaccinated with bivalent vaccine (ND, IB),

G4: Group 4 vaccinated with bivalent vaccine (ND, IB),

G5: Group 5 infected with MG then vaccinated with bivalent vaccine (ND, IBD),

G6: Group 6 vaccinated with bivalent vaccine (ND, IBD),

G7: Group 7 infected with MG then vaccinated with REO vaccine, G8: Group 8 vaccinated with REO vaccine.

Discussion

In fact, vaccines play a significant role in the prevention and management of poultry diseases throughout the world. Their usage in the industry of poultryhas historically been intended to prevent or minimize the occurrence of clinical disease at the farm level and hence increase productivity. The availability of vaccinations and immunization programs varies widely based on a number of local circumstances (such as the type of production, biosecurity degree, local disease pattern, maternal immunity statusand potential losses). the poultry industry usually oversees vaccination of poultry but, it is used only sporadically as part of a national or regional disease eradication effort to eliminate a few significant chicken diseases (such as AI and ND).The effectiveness of the vaccination program once it has been established should generally be modified and adjusted based on local characteristics that may influence the strategy, the design, and the immunization schedule. The type of poultry production (such as commercial or rural), the organization of the industry (such as vertical integration), and the densities of various bird species are only a few of the various considerations that should be made as the current state of the disease, the accessibility of vaccinations; the use of other vaccines; the resources available (e.g. manpower and equipment); the costs involved; the presence of other diseases**(Marangon and Busani, 2006).**

Presence of other microorganisms such asMycoplasma contaminated vaccine or Mycoplasma infection of hostmay produce varying degree ofimmunological response**Fathy et al. (2017).** Also**, Ishfaq et al. (2020)**stated that Mycoplasma gallisepticum strains differ in virulence and infectivity, and infection might occasionally be imperceptible and goes undetected**.**Before starting a control program, it is useful to knowthe prevalenceof microorganisms in different poultry species in the area involved(**Gole et al.,2012)**.It predisposes birds to the action of some vaccine strains, such as ND or IB, and other respiratory viruses (**Stipkovits et al.,2012).** In line with current studyfindingthe results supported the observation of **Bolha et al. (2013)** when mildly virulent virus strains infected birds that had already been infected withMycoplasma, the interactions between the host,Mycoplasmacells, and virus could result in effects that differ from those of single pathogen infection.The goal of this current work to demonstrate whether Mycoplasma infection affected the efficacy of some inactivated bivalent and monovalent poultry viral vaccines and to assess the protection provided by vaccination against viral challenge.Experimental co-infection of MG with avian influenza leads tosever clinical signs and reduced weight gain**(Stipkovits et al.,2012).**

Respiratory infections are a common cause for increased mortality rates in poultry worldwide. Circulating pathogens need to be identified and further characterizedto improve intervention strategies. It was not known which pathogens contribute to the high mortality associated with respiratory disease,the main pathogens are Mycoplasma gallisepticum(MG), Mycoplasma synoviae(MS), avian influenza virus (AIV), and infectious bronchitis virus (IBV) in chicken and turkey flocks as multi-infections(**Sid et al.,2015)**. Infection with MG may result ina variety of respiratory signs including coughing, sneezing, and nasaldischarge (**Naylor et al.,1992)**. AIV has been found to be involved inmulti-causal respiratory infections where interaction with Mycoplasma was thought to be responsible for high mortality (**Bano et al.,2003)**,additionally, interaction of IBV with pathogens including MG(**Leighet al.,2012)**.

Combining a chicken-passage mild IB vaccine virus with MSincreased the incidence of air sacculitis compared to a non-passage virus vaccine, so that chicks vaccinated with IB combined with MS vaccine have higher antibody (AB) titers and are adequately protected against IBV disease alone as proposed by**Hopkins and Yoder(1984)**.The study concluded that IB infection could cause latent Mycoplasma infection and highlighted the importance of using Mycoplasma-free chickens when testing the efficacy of viral vaccines.The immune system of chickens is negatively impacted by all known pathogenic mycoplasma infections. Unfortunately, at the time of NDV vaccination, the immunomodulatory impact of MG has not yet been discovered. **Naglaa (2019)**compared to the non-vaccinated non-infected chicken group, the MG and mixed infection groups with

MG and Ecoli showed a significant increase in antibody titer from 28 to 35 day old, whereas the MG and mixed infection groups with MG and Ecoli showed no significant elevation in NDV antibody ELISA titer over time.

M. gallisepticum can prevent phagocytosis and interfere with B and T lymphocyte operations. Mycoplasma lipoproteins interact with the host immune system in which M. gallisepticum was reported to enter non-phagocytic cells, such as chicken erythrocytes, it induces the expression of various enzymes and cytokines (lymphoactin, CXCL13, CXCL14, RANTES, MIP-1b, IL-1b, and IFN-g) that aid in the development of local tissue lesions.Mycoplasma-infected chickens are susceptible to subsequent bacterial infections. Chickens are more vulnerable to various illnesses because of M. gallisepticum (**Stipkovits et al.,2012)**. Another study reported thatMycoplasma gallisepticummodulate host immunesystem through activation of toll-like receptors (TLR)including TLR-2/TLR-4/TLR-6, nod-like receptors,and NF-kB pathway**(Li et al., 2019; Chen et al., 2020)**

Mycoplasmas can affect the cell-mediated immune system by suppressing (in the chronic phase of infection) or stimulating (in the acute phase of infection) B and T lymphocytes (**Gaunsonet al.,2000)**, and this can explain the immune stimulating effect ofMycoplasmaas seen in the current experiment that represents the acute phase of infection where MG increased antibody titers significantlyagainst ND antigen in infected vaccinated groups (G1, G3 and G5) (7.7 log_2, 7.8 log_2, 7.9 log_2, respectively) 4 weeks post vaccination significantly compared to non-infected groups (G2, G4 and G6) (6.5 log_2, 6.7 log_2, 7.1 log_2, respectively), as these findings agreed with those of **Silva et al.(2008)** who found that MS infection followed by ND vaccination seven days later resulted in higher and longer lasting serologic responses to ND vaccine in non-MS infected chicks compared to infected and vaccinated chicks.

The effects of MG and MS on the antibody response to ND vaccination remain unknown, also the MG pre-infected ND vaccinated groups (G1, G3 and G5) were more protected (100%, 90%, 100%, respectively) than the non-infected NDVV vaccinated groups (G2, G4 and G6) (90% for each), according to the protection percentage against NDVV as shown in Table(2).The non-vaccinated non-infected birds (G9), however, were extremely vulnerable to the VVNDV challenge and had overt clinical signs. Clinically, they showed indications of depression, anorexia, comb and wattle cyanosis, and respiratory indicators such gasping and rales.The present study also agree with **Fathy et al.(2017)** whoconcluded that Influence of MG on the serological immune response to inactivated viral monovalent vaccinations of chicken is significant and obvious.

The findings of this study examine the potential reasons why some viral poultry vaccines may produce erroneous results during testing, particularly when chicken flocks have a history of Mycoplasma infection, also elevated antibody titers by ELISA test against IBV, IBDV, and Reo virusthat concurred with this currentfindings.At 28 days post-vaccination, the Mycoplasma pre-infected group (G1) had the significant increased titer of AI antibodies by HI test, which was 7.1 log_2, whereas the AI vaccinated non-infected group (G2) had the lowest titers 6.5 log_2. These findings corroborated those made by **Thacker et al. (2001)** who employed anrespiratory experimental model to study M. hyopneumoniae and swine influenza virusinteraction.The protection % inthe MG pre-infected and vaccinated group(G1) was (90%) while in non infected and vaccinated group(G2) was (80%) as mentioned in Table (2), figure (1).The release of proteases enzymes during the replication of bacteria like MG may be the cause of the increased pathogenicity as reported by **Subtain et al.(2016).**In challenged chickens the most pathognomonic signs in unvaccinated group (9)waswattleand comb cyanosis, echymosis on the shanks, facial edema, greenish diahrria and nervous signs

Also, the present study demonstrated that IB antigen detection by ELISA in day 28 post vaccination in the MG pre-infected vaccinated group(G3) S/P ratio of (1.7) and protection % of (90%) that was not significantly higher than the MG non-infected and vaccinated group (G4) (0.6)S/P ratioand protection % of

(90%) as detailed in Table (3), figure (2).Affected birds in group (9) revealed congested trachea, caseous and bloody plugs at the tracheal bifurcations, as the protection % was (0%).The MG pre-infected and vaccinated group(G5) containing IBD antigenS/P ratio was (1.7) which not significantly increased4- weeks after vaccination and the protection % was (100%) while in non-infected group (G6),S/P ratio was (0.8)and the protection % was (90%). For group (9) the protection was (0%),petechiae and echymotic hemorrhages of the thigh and pectoral muscles, a much enlarged and hemorrhagic bursa of Fabricius, a hemorrhagic mucosa between the proventriculus and ventricular junction, and hemorrhagic caecal tonsils were all seen in the infected birds.

In comparison to a single infection, Mycoplasma with multiple viral infections has shown more severe clinical presentation (**Sly and Jones,2011)**.Few studies have looked into how Mycoplasma species and APEC affect chickens who have received the NDV vaccine. The decreased detectable hemagglutination (HI) and IgG antibody titers provided proof of**Hassanin et al. (2014)**. Moreover, in Brazil, previously M. synoviae-infected chicks showed weaker serological responses to the NDV vaccine **Silva et al., (2008)**.

Naglaaet al. (2019) who made a single and mixed infections with (MG) and/or (E. coli) and cited the effects on the chicken immune response elicited by (NDV) vaccine, which theyused to corroborate the current studyfindings. The research focused on the immunosuppressive properties of MG and E. coli infection in chickens who have received an NDV vaccine.

Al-Afaleq et al. (1989) stated that in the dually infected group, neutralizing antibodies byReo viruswere found after 3 weeks and remained until the experiment's end at 15 weeks (Reo virus together with the MS combined infection).**Reck et al. (2012)**described the histopathological alterations brought on by combination MS and avian ortho-Reo virus infection in broilers. The presence of a synergistic interaction between MS and the avian ortho-Reo virus was revealed by mixed infection.

The Reo highest titer of antibodiesby ELISAtest was at 28 dayspost-vaccinationinMGpre-infected Reo vaccinated group(G7) (0.3)not significant and a protection% (100%), compared to non-infected vaccinated group (G8) with S/P ratio of (0.28)and a protection% (100%)in which the results agreed with **Fathy et al. (2017**). The control group (9) challenged with Reo virus revealed a straw-colored fluid has built up in the joint space along with enteritis and oedema in the vicinity of the hock joints.

Different ages have a significant impact on the prevalence of M. synoviae infection in chickens. According to **Xue et al. (2017)**, rates increased with increasing age, with 1- to 3-day-old chicks reporting sero-prevalence from 29 to 54%, and 2.70 to 5.65% for 3- to 4-week-old chickens, 35-week-old chick survival rates ranged from 71 to 83%**Veronica et al. (2021)**considered that the positive rates observed appeared to rise with sample age according to PCR data, prevalence in layers was 95% and in broiler breeders was 35%. It is necessary to have a deeper understanding of chicken immunological response to MG infection. According to **Wang et al. (1990),** MG was identified as nonpathogenic, isolated from chickens' respiratory and reproductive tracts, and assessed as a potential vector for MG antigen expression by**Evan et al. (2005).**Moreover,Mycoplasma-infected chickens are susceptible to subsequent bacterial infections. It's been established that in addition to Mycoplasma infection, E. coli can spread more quickly and cause bacteremia, severe air-sacculitis, and peritonitis. M. gallisepticum is also known to decrease chickens immunological reaction to Haemophilus gallinarum. Additionally, it has been shown that chickens with compromised immune systems, in which T-cell-mediated immunity is reduced, are more susceptible to contracting the LPAI virus**Stipkovits et al. (2012)**.

More research is required to show the negative impact of those illnesses on the immune system at the time of vaccination as a result of these information gaps.In this work, serological immune responses were used to highlight the outcomes of chicken immunization with inactivated viral vaccines, particularly in the presence or absence of Mycoplasma infection in chicks. There is evidence that when compared to vaccine-treated flocks without MG infection, flocks of chickens with MG infection may yield inaccurate results.

Conclusion

This workdesigned to demonstrate the differences in the efficiency of different inactivated poultry vaccines both (monovalent and bivalent) that utilized in field by different routine tests evaluation comparing the results in presence or absence of Mycoplasma gallisepticum selecting prevalent and updated challenge viruses strains according to different vaccines antigens and focused on the incidence of false results to chicks flocks that previously infected with Mycoplasma gallisepticum which can be a main factor for the significant increase in antibody titers (G1, G3 and G5) (7.7 $\log_2$, 7.8 $\log_2$, 7.9 $\log_2$, respectively)for ND,(7.1 $\log_2$)for AI antigen (G1), andS/P ratio (G3, G5 and G7) was (1.7, 1.7, 0.3) for IBV, IBDV, and Reo virus antigens respectively28 days post-vaccination for the pre-infected groups. while thelowest titer appeared in non-infected vaccinated groups (G2, G4 and G6) (6.5 $\log_2$, 6.7 $\log_2$, 7.1 $\log_2$, respectively)for ND, (6.5 $\log_2$)for AI antigen, andS/P ratio (G4, G6 and G8) was (0.6, 0.8, 0.28) for IBV, IBDV, and Reovirus antigens respectively. The study propose the need to take in consideration the different precautions needed to prevent exposure to Mycoplasma gallisepticum indifferent farms or research laboratories during the evaluation process. Moreover, selecting suitable vaccines in the predisposing farms to Mycoplasma infection.

Ethical approval

The study was approved by Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo, Agricultural Research Center (ARC), Giza, Egypt. All efforts were made to maintain ethics and humane handling of chicks according to the ethical standards guidelines released by Cairo University concerning animal welfare.

Study period and location

The study was conducted in March 2022 at (CLEVB), Abbasia, Cairo, Egypt..

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