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Original Article

Assessment of mycoplasma vaccine efficacy in reducing infection with Newcastle disease virus

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The present work recorded the impact of using *Mycoplasma gallisepticum* vaccines on post-vaccinal response and protection against challenge with Newcastle disease virus. Specific pathogen-free chickens were divided into eight groups of forty chickens each. Group G1 was vaccinated with *Mycoplasma gallisepticum* live attenuated and *Mycoplasma gallisepticum* inactivated vaccines. Group G2 was vaccinated with *Mycoplasma gallisepticum* live attenuated, *Mycoplasma gallisepticum* inactivated and Newcastle disease inactivated vaccines. Group G3 was vaccinated with *Mycoplasma gallisepticum* live attenuated vaccine. Group G4 was vaccinated with *Mycoplasma gallisepticum* live attenuated and Newcastle disease inactivated vaccines. Group G5 was vaccinated with *Mycoplasma gallisepticum* inactivated vaccine. Group G6 was vaccinated with *Mycoplasma gallisepticum* inactivated and Newcastle disease inactivated vaccines. Group G7 was vaccinated with Newcastle disease inactivated vaccine. Group G8 was kept as non-vaccinated control. The Newcastle disease hemagglutination inhibition antibodies and mortality percentages were measured. Group G7 recorded the best protective Newcastle disease hemagglutination inhibition antibody titer (7 $\log_2$). Group G2 recorded a marginal satisfactory antibody titer (6 $\log_2$) after vaccination by the three tested vaccines. The remaining groups revealed unsatisfactory titers ranged from 0-5. The protection levels for G2, G4, G6 and G7 ranged from 70% to 100%, but only G2 and G7 were considered protected. G1, G3, G5 and G8 showed typical clinical signs of Newcastle disease. The *Mycoplasma gallisepticum* vaccines couldn't improve the response to Newcastle disease inactivated vaccine. The results suggest that *Mycoplasma gallisepticum* vaccination is immunosuppressive rather than immunomodulatory in Newcastle disease vaccination.

Keywords: *Mycoplasma gallisepticum*; Newcastle disease virus; vaccines; mortality; virus shedding.

Introduction

Newcastle disease (ND) is a viral disease of poultry caused by Newcastle disease virus (NDV), a single-stranded RNA avian paramyxovirus type 1. The disease is present worldwide and infects most bird species, causing huge losses in the poultry sector. In developing countries, outbreaks of ND have occurred in many areas, resulting in severe economic and commercial losses. It is an endemic disease in Egypt.[1]

The primary NDV control strategies depend mainly on vaccination with live attenuated or inactivated vaccines.[2] In United States, during the outbreak of California in 2002–2003, about 2,500 premises (4 million birds) were depopulated. Losses were estimated at $162 US million. In 2008, the World Organization for Animal Health (OIE) considered ND with certain virulence criteria, a notifiable disease.[3]

Mycoplasma gallisepticum (MG) is a bacterial pathogen which causes chronic respiratory disease

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(CRD) with economic losses.[4] It was listed by the OIE as a primary cause of CRD of poultry.[3] The infection usually produces mild symptoms with the ability to synergize with other avian respiratory agents such as infectious bronchitis virus, NDV and *Escherichia coli*, therefore mycoplasmosis is considered an economically important disease.[5] Poultry producers depend mainly on vaccination and biosecurity to manage mycoplasmosis.[6]

MG induces immunosuppressive effects by damaging the immune system and affecting B and T cells development, leading to an impairment of chicken immune system. In turn, it leads to down-regulation of the post-vaccination immune response and consequently results in limited development of protection. Earlier reports demonstrated the immunosuppressive effect of MG in chickens vaccinated with NDV vaccine. This was confirmed by the reduction of hemagglutination inhibition (HI) titer and IgG antibody titers against NDV,[7] as well as delayed cytokine response initiation. Additionally, there is a lack of information concerning the immune response to mixed MG and NDV vaccines in Egypt. Studies are needed to evidence the adverse effects of MG vaccination on the immune system at the time of ND vaccination.

The present study was conducted to evaluate the most protective and effective vaccination program against ND and GM vaccines, in order to contribute to the optimization of vaccination programs to achieve the best flock immune status.

Materials and Methods

Vaccines

Three commercial vaccines were used in the current study: a live attenuated MG vaccine, an inactivated MG vaccine and an inactivated ND vaccine.

Virus

A virulent NDV local isolate type (genotype 7): 7 NDV -B7-RLQP-CH-EG-12, accession No KM288609 was supplied by the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo, Egypt which has been routinely used at CLEVB for challenge testing.

Specific pathogen free chickens and eggs

Three hundred and twenty, 5-weeks old specific pathogen free (SPF) chickens and 9 days old embryonated chicken eggs (ECE) were obtained from the SPF Egg Production Farm, Koum Osheim, El-Fayoum, Egypt.

Blood samples were collected from all groups prior to vaccination to assure its freedom of MG and NDV, to be used as negative control for further investigations.

Experimental designs

Eight groups of SPF chickens, 40 chickens per each, were vaccinated and challenged at the following time intervals (Table 1).

Table 1. Vaccination-challenge groups design.

Group	Age of Vaccination			NDV challenge age
	5 weeks	8 weeks	12 weeks	
G1	MG live attenuated	MG inactivated		12 weeks
G2	MG live attenuated	MG inactivated	ND inactivated	16 weeks
G3	MG live attenuated			9 weeks
G4	MG live attenuated	ND inactivated		12 weeks
G5		MG inactivated		12 weeks
G6		MG inactivated	ND inactivated	16 weeks
G7	ND inactivated			9 weeks
G8		Control		16 weeks

Vaccination and boosting

As shown in Table 1, at 5 weeks old, SPF chicks in G1, G2, G3 and G4 were immunized with live attenuated MG vaccines and G7, with inactivated ND. Three weeks later (8 weeks old), G1 and G2 were boosted with inactivated MG; G4, with inactivated ND and G5 and G6 were vaccinated with inactivated MG. At 12 weeks old, G2 and G6 were vaccinated with inactivated ND. All vaccines were administered by the recommended route (subcutaneous) and dose (0.5mL).

Serum plate agglutination

Blood samples were collected on 28th day after last immunization for serological testing by serum plate agglutination (SPA) test to assure formation of protective antibody against MG.[3]

Hemagglutination inhibition test

The collected serum samples of all experimental groups were also tested to determine the NDV antibody titer of each vaccinated group by HI test as mentioned in OIE.[8] Two fold serial dilutions of serum samples were applied from 1/2 to 1/2048 against 4 HA units of ND antigen 10^6 EID_{50}/0.1 mL by HA test.[8] The geometric mean of ND HI antibody titer was calculated.[8,9] The mean HI titer has to be not less than 6 $1og_2$.

Challenge test

Ten birds from each group were challenged (at different weeks according to the group, Table 1) with 10^6 LD_{50} NDV(7 NDV-B7-RLQP-CH-EG-12, accession No KM 288609), 1 mL/ bird, and were inspected for further 6 days for clinical signs and mortalities.[8,10] Virus shedding post challenge was shown by tracheal swabs collected on days 1, 3 and 5 from live birds.[11] All NDV swabs were titrated using 9 day old SPF ECE;[12] virus shedding was calculated using Kärber method.[9] At the end of observation days, live birds with moderate to severe signs were humanly euthanized for detection of ND post-mortem gross lesions.[13] NDV protection percentage has to be 90% or higher.

Ethical approval

All animal related procedures were applied with relevant guidelines and regulations of Veterinary Cairo University Institutional Animal Care and Use Committee (Vet.CU-IACUC), according to local Egyptian laws. The study was approved ethically by CLEVB, Cairo, Egypt.

Results

Serum plate agglutination of *Mycoplasma gallisepticum* vaccinated groups

All sera from the MG vaccinated groups showed positive results by serum plate agglutination test.

Hemagglutination inhibition titers of Newcastle disease vaccinated groups

The mean HI titer was calculated for all experiment groups (G1-G8) after blood samples were collected separately from each vaccinated group before the challenge test. The G7 immunized with ND vaccine (at 5 weeks) recorded the best protective ND antibody titer (7 log_2). Also, G2 recorded a marginal satisfactory antibody titer (6 log_2) after vaccination with the three tested vaccines. The remaining groups revealed unsatisfactory titers ranging from 0-5 (Table 2).

Post challenge protection rate

The protection level was evaluated according to the percentage of mortality, morbidity and virus shedding after inoculation of tracheal swabs into 9-day-old SPF ECE. The groups vaccinated with ND showed highest protection rates as follow: 90%, 80%, 70% and 100% for groups G2, G4, G6 and G7, respectively. Only G2 and G7 were considered protected (Table 3).

Table 2. Newcastle disease virus hemagglutination inhibition titers of vaccinated groups.

Group	G1	G2	G3	G4	G5	G6	G7	G8
HI titer log_2	0	6	0	5	0	4.1	7	0

Table 3. Protection percentages against virulent NDV post challenge.

Groups	n= 10		
	Positive	Negative	Protection (%)
G1	8	2	20
G2	1	9	90
G3	8	2	20
G4	2	8	80
G5	10	0	0
G6	3	7	70
G7	0	10	100
G8	10	0	0

Fig. 1. Post challenge lesion, post mortem inspection, showing pin pointed hemorrhages at the proventriculus.

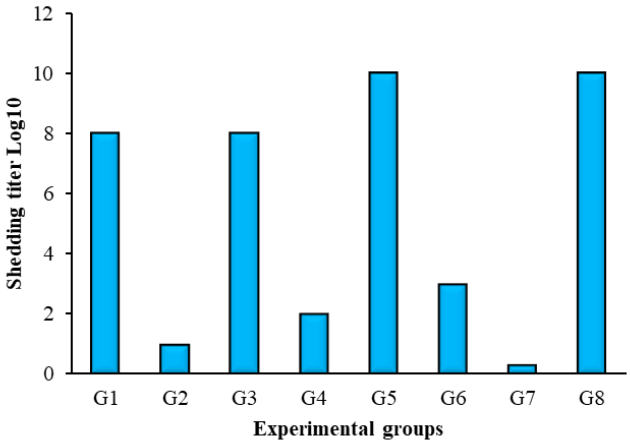

Fig. 2. NDV shedding post challenge with virulent Newcastle disease virus.

Groups G1, G3, G5 and G8 showed typical clinical signs of NDV, mainly nervous manifestations with nasal and ocular discharge. At post mortem (PM) inspection, the same groups showed characteristics of NDV infection, such as punctate hemorrhages in the proventriculus and cecal tonsils, with congested trachea and catarrhal exudates (Fig. 1).

The results of NDV shedding post challenge were consistent with mortality and morbidity rates (Fig. 2). Groups immunized with ND vaccines (G2, G4, G6 and G7) showed low or no virus shedding post challenge.

Discussion

Over the past years, studies have been conducted on immunosuppressive effect of bacteria at the time of ND, which may render vaccine efficacy by affecting the immune response against ND vaccine.[14] The current study evaluates the impact of using MG vaccines for chicken immunization before vaccination with ND vaccine, for this purpose, the humoral immune response by detection of antibodies anti-hemagglutinin protein using HI test and the ND vaccine protective efficacy after challenge test were evaluated.[15]

The antibody titer of the ND vaccinated groups is directly proportional to the immunogen retention time. The present study showed that the humoral immune response against NDV decreased in all MG vaccination models. Low titers of antibodies against NDV in birds vaccinated with either live attenuated or inactivated MG confirmed its immunosuppressive effect.[16]

The present work also studied the impact of using live attenuated and inactivated MG vaccination on NDV experimental infection. The immune response of all vaccinated groups was evaluated; sera from groups G2, G4, G6 and G7 were especially considered, since these chickens were vaccinated with NDV inactivated vaccine, in addition to different MG vaccines in several schedules.

Groups vaccinated with inactivated or live attenuated MG vaccines and ND vaccine revealed lower NDV antibody titers compared to the group immunized with ND vaccine only. This finding may be explained by the ability of the ND vaccine to induce specific and non-specific immune responses against NDV, which

corresponds to the development of protective NDV antibody titers when the ND vaccine was used. Also, an adequate amount of non-specific factors, such as cytokines like interferon gamma, which activate the production of antigen-stimulated B lymphocytes, cytotoxic T lymphocytes, macrophages and natural killer lymphocytes, could be produced.[17]

On the other hand, groups vaccinated with inactivated or live attenuated MG vaccines before ND vaccination resulted in a marginal NDV antibody titer. This could be attributed to defective B-lymphocyte production in the live attenuated MG vaccinated group.[18] In addition, IgM to IgG switching could not have occurred when the inactivated MG vaccine was administered, but could be possible when the live vaccine was used. Furthermore, interferon gamma may not be efficiently produced, which in turn leads to inadequate macrophage activation.[17]

Mycoplasma vaccines may induce an immunosuppressive effect by affecting B and T cells progress, leading to a drastic impairment of the immune system, accompanied by down regulation of post-vaccinal immune response to ND and the development of a very limited post-vaccination protection against ND. These findings were evidenced by the low level of antibody titers under the effect of MG vaccination.[14]

The current work revealed that the level of humoral immune response to NDV was decreased with both MG vaccines. The low NDV antibody titers in groups vaccinated with MG and ND vaccines confirmed to be immunosuppressed. This immunosuppression led to a negative impact on ND vaccination. This information can be a guidance when design an appropriate vaccination strategy for the prevention and control of NDV.[14]

Both live attenuated and inactivated MG vaccines produced an adverse effect on ND vaccine. Unsatisfactory HI titers suggest its negative impact.[19] Unfortunately, there is no evidence of immunomodulatory effect of MG vaccination before NDV vaccination.

Additionally, a challenge with virulent NDV was applied to measure the protective immunity induced against NDV. It is evident that the mortality rates of birds vaccinated only with ND vaccine were very low after challenge. Only birds from G2 and G7 were protected (90% and 100%, respectively) when protection percentages and shedding titers were calculated. Groups vaccinated with MG live attenuated/ND vaccines (G4) or MG inactivated /ND vaccines (G6) showed an impotent protection percentage, but still better than ND non-vaccinated groups (G1, G3, G5). This finding supports previous reports of the negative effect of MG vaccines on the inactivated ND vaccine, since vaccinating only with the ND vaccine was the best variant of those studied in the experiment, the birds were able to persist the challenge without mortality, PM lesions, shedding or clinical signs.[19]

On the other hand, the lower protection levels demonstrated insufficient immunity to protect chickens against NDV, which can be attributed to the immunosuppressive effect of using MG vaccines before inactivated ND vaccination.[14]

Conclusions

MG vaccination did not improve the response to ND vaccination, suggesting an immunosuppressive rather than immunomodulatory effect.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author´s contribution

Hala Mahmoud: performed the experiments and wrote the manuscript.

Marwa Fathy Elsayed: performed the experiments and wrote the manuscript.

Reem A. Soliman: performed the experiments.

Mounir El Safty: designed the experiments.

Moustafa A. Zaghloul: performed the experiments.

All authors reviewed and approved the final version of this manuscript for publication.

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Evaluación de la eficacia de la vacuna contra micoplasma para reducir la infección por el virus de la enfermedad de Newcastle

Resumen

En el presente trabajo se registró el impacto de la utilización de vacunas contra *Mycoplasma gallisepticum* sobre la respuesta posvacunal y la protección frente al reto con el virus de la enfermedad de Newcastle. Pollos libres de patógenos específicos se distribuyeron en ocho grupos de cuarenta pollos cada uno. El grupo G1 se vacunó con vacunas vivas atenuadas e inactivadas contra *Mycoplasma gallisepticum*. Al grupo G2 se le aplicaron las vacunas: viva atenuada contra *Mycoplasma gallisepticum*, inactivada contra *Mycoplasma gallisepticum* e inactivada contra la enfermedad de Newcastle. El grupo G3 se inmunizó con la vacuna viva atenuada contra *Mycoplasma gallisepticum*; el G4, con las vivas atenuadas contra *Mycoplasma gallisepticum* e inactivada contra la enfermedad de Newcastle; el G5, con la vacuna inactivada contra *Mycoplasma gallisepticum*; el G6 con las vacunas inactivadas contra *Mycoplasma gallisepticum* y la enfermedad de Newcastle; el G7, con la vacuna inactivada contra la enfermedad de Newcastle y el G8 se mantuvo como control no vacunado. Se midieron los anticuerpos de inhibición de la hemaglutinación contra el virus de la enfermedad de Newcastle y los porcentajes de mortalidad. El grupo G7 registró el mejor título de anticuerpos inhibidores de la hemaglutinación contra la enfermedad de Newcastle (7 $\log_2$). El grupo G2 registró un título de anticuerpos marginalmente satisfactorio (6 $\log_2$) tras la vacunación con las tres vacunas ensayadas. Los demás grupos revelaron títulos insatisfactorios que oscilaban entre 0 y 5. Los niveles de protección de los grupos G2, G4, G6 y G7 oscilaron entre el 70% y el 100%, pero sólo G2 y G7 se consideraron protegidos. Los grupos G1, G3, G5 y G8 mostraron signos clínicos típicos de la enfermedad de Newcastle. Las vacunas contra *Mycoplasma gallisepticum* no pudieron mejorar la respuesta a la vacuna inactivada contra la enfermedad de Newcastle. Los resultados revelan que la vacunación con *Mycoplasma gallisepticum* es más inmunosupresora que inmunomoduladora en la vacunación contra la enfermedad de Newcastle.

Palabras clave: *Mycoplasma gallisepticum*; virus de la enfermedad de newcastle; vacunas; mortalidad; esparcimiento de virus.

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