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Original Article

Evaluation of efficacy, cell mediated and humoral immune response of two different mycoplasma vaccines in specific pathogenic free chickens

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To investigate cell mediated and humoral immune response following vaccination with live mycoplasma vaccines, 100 specific pathogenic free chickens were inoculated with two different live mycoplasma vaccines: *Mycoplasma gallisepticum* (ts-11) and *Mycoplasma synoviae* (H); another 50 chickens were kept as controls (non vaccinated chickens). To evaluate the cell mediated immune response, peripheral blood leucocytes were obtained from vaccinated and control chicken groups and the lymphocyte proliferative response and nitric oxide level were determined. The immunomodulatory cytokines expression profiles elicited by the vaccines were evaluated; the mRNA expression level of interleukin-6 and gamma interferon were determined in spleen of vaccinated specific pathogenic free chickens. In parallel, sera collected from vaccinated and control chickens were examined using an enzyme-linked immunosorbent assay antibody kit to assess the humoral immune response. A challenge test was applied at 4th week post vaccination to all vaccinated chickens and controls against virulent strains of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. The results confirm the presence of consistent lymphoproliferation with production of interferon and nitric oxide *in vitro* in the two vaccinated chicken groups compared to the negative control. Moreover, the tested vaccines induced high seroconversion level with satisfactory protection (%) at 4th week post vaccination. It was concluded that the two live mycoplasma vaccines can protect the vaccinated chickens against *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infections suggesting a significant role for cell-mediated immunity.

Keywords: cell-mediated immunity; humoral immune response; interleukin-6; mycoplasma infections; vaccines.

Introduction

Mycoplasmosis is one of the important bacterial diseases that affects chickens and turkeys (broiler and layer). This disease causes respiratory signs, drop in egg production, decrease egg quality, high embryonic mortality, poor hatchability, high morbidity, poor weight gain and increased medication costs.(1) Among pathogenic mycoplasmas, *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are the most important causative agents of mycoplasmosis.(1) These organisms are classified into the class *Mollicutes*, family *Mycoplasmataceae*.(1,2) Mycoplasmas are small prokaryotes that differ from other bacteria in not having cell wall.(2) MG is the most economically significant and affects, mainly, gallinaceous avian species. It causes chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys.(2) MS is also pathogenic for both chickens and turkeys. All age groups of turkeys and chickens are susceptible, but disease is more common in commercial layer chicken up to 32 weeks old.(3)

Vaccination and biosecurity measures are considered an effective tool for controlling MG and MS in poultry.(4) Concerning live MG vaccine, three commercial live

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vaccines (F strain vaccine, 6/85 strain vaccine, and ts-11 strain vaccine) are available for use in poultry with high degree of efficacy in chickens, but not suitable for use in turkeys. Especially, live ts-11 vaccine has been widely used to control and even eradicate MG infection in commercial chicken flocks; it can be administered via ocular route and induces long lasting immunity 3 months post vaccination.[5] Moreover, this vaccine can stimulate a detectable and systemic antibody response. Live vaccination against MS is not frequent in practice and the only commercially available vaccine is MS-H.[3] Potency of this type of vaccine is evaluated routinely by a vaccination-challenge test (efficacy) or a serological response test to vaccination (immunogenicity) in specific pathogenic free (SPF) chickens following the international protocols.[6]

Besides the humoral immunity, the role of cell mediated immune response in protection of chickens vaccinated with live mycoplasma vaccines is still unclear. Therefore, evaluation of the cell mediated immune response and its indictors are mandatory; for this purpose, determination of lymphocyte proliferation and estimation of the immunomodulatory cytokines expression profiles elicited by the vaccine, the mRNA expression level of interleukin-6 (IL6) and gamma interferon (IFNγ) are necessary.[7] IL-6 is a multifunction cytokine that has been shown to be necessary for T cell differentiation, and also involved in antibody formation as a B-cell differentiation factor.[8] IFNγ is a cytokine that belongs to IFN type 2 and its genes are located on chromosome 12; the gene (IFNGR1) encodes the ligand-binding chain (alpha) of the heterodimeric IFNγ receptor, which is found in macrophages.[9] It stimulates T cell differentiation for the T helper 1 (Th1) type response and clonal expansion. Nitric oxide (NO), a free radical that acts as a pro-inflammatory cytotoxic mediator and is produced by the inducible nitric oxide synthases (iNOS) in activated macrophages and neutrophils, regulates a number of immunological and physiological processes.[10] This study was aimed to evaluate the cell mediated and humoral immune response of two commercial live mycoplasma vaccines: ts-11 (against MG) and MS-H (against MS) in SPF chickens.

Materials and Methods

Specific pathogenic free chicks

A total of 150 one-day-old SPF chickens were obtained from Khom Oshem farm, El Fayoum, Egypt. They were reared and housed in positive pressure stainless steel isolation cabinets with continuous light exposure.

Vaccines

Imported commercial live MG ts-11 vaccine

Vaxsafe® MG, batch: MGS192851BG. It was administrated via ocular route at a dose of 0.03 mL/ bird.

Imported commercial live MS -H vaccine

Vaxsafe® MS, batch: MSH200321BG. It was administrated via ocular route at a dose of 0.03 mL/ bird.

Experimental design

One hundred and fifty SPF chickens were used to evaluate the two live commercial vaccines; they were divided into three subgroups as follows:

Group 1 (50 SPF chickens): vaccinated with live MG vaccine (ts-11)

Group 2 (50 SPF chickens): vaccinated with live MS -H vaccine.

Group 3 (50 SPF chickens): kept as non-vaccinated group (control -ve).

Ten and five SPF chickens from each of the vaccinated and control groups, respectively, were bled by wing vein puncture with heparin (20 IU/mL) at 0, 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th days post vaccination (DPV) to determine lymphocyte proliferation assay and NO. Spleen tissues from euthanized chickens were examined at 1st, 3rd, 5th, and 9th DPV using quantitative real time-PCR (qRT-PCR) to determine IL-6 and IFNγ**.** Blood samples were collected from all groups at 1st, 2nd, 3rd and 4th week post vaccination (WPV). Collected antisera were tested for detection of MG and MS antibodies using ELISA test. Challenge test against virulent strain of MG was performed at 4th WPV in 25 vaccinated and 10 control SPF chickens. The same

numbers of vaccinated and control chickens were challenged at 4th WPV using virulent MS infection.

Evaluation of cell mediated immune response

Detection of lymphocyte proliferation

A lymphocyte proliferation assay kit was used: XTT (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium cell proliferation assay kit, cat. no. 30-1011K (1000 assays), American Type Culture Collection, United States.

Chicken blood lymphocytes were separated.[11,12,13] Lymphocyte suspension was adjusted to $5x10^6$ cells/mL, suspended in RPMI containing 10% fetal calf serum (FCS) and seeded in a 96-well plate with negative (unstimulated cultures containing only the medium) and positive controls (a T cell mitogen, phytohemagglutinin, at a concentration of 25 g/well) followed by incubation during 48 h at 37°C. The activated XTT solution (50 µL) was added to each well, then the plate was incubated in a 5% CO_2 incubator at 37°C for 4 h before obtaining readings. Absorbance was measured at 490 nm using a spectrophotometer with blank wells used for background control.

Determination of IL-6 and IFNγ in spleen tissue by quantitative real time-PCR

Total RNA was isolated from spleen of vaccinated and non-vaccinated chickens throughout the course of the experiment, using RNeasy Mini Kit (Catalogue no.74104, QIAGEN Inc., Valencia, CA, USA) and DNase treatment with QIAGEN®™ RNA purification kits to purify RNA from DNA contamination. RNA in each sample was quantified using NanoDrop-1000 (Thermoscientific, Wilmington, DE). The qRT-PCR was performed using Quantitect probe RT-PCR (Metabion, Germany) according to the manufacturer's recommendations. Primers and probes selected for the amplification of 28SrRNA, IL6 and IFNγ are shown in Table 1. The qRT-PCR runs were performed using 7500 Real-Time PCR System (Stratagene MX3005P) with the same PCR conditions for each targeted gene of different cytokines: 30 min at 50°C (reverse transcription), 95°C for 10 min (primary denaturation), followed by 40 cycles of 94°C for 15 s (secondary denaturation), and 60°C for 1 min.

Amplification curves and Ct values were determined using the stratagene MX3005P software. To estimate the variation in gene expression in the RNA of the different samples, the Ct of each sample was compared with that of the positive control group using the "$-2^{\Delta\Delta Ct}$" method.[16]

Whereas ΔΔCt = ΔCt reference – ΔCt target; ΔCt target = Ct control – Ct treatment; ΔCt reference = Ct control-Ct treatment; E: efficiency of amplification.

Measurement of nitric oxide level

Monocytes were isolated from pooled buffy coats of vaccinated chickens and incubated at 37°C for 2 h, then the non-adhered cells were discarded. Differentiation of monocytes into macrophages was carried out by culturing for 3-5 days with 10% FCS. Zymosan (5mg/

Table 1. Oligonucleotide primers and probes used in real time PCR.

Gene	Primer sequence (5'-3')	Reference
IL6	GCTCGCCGGCTTCGA GGTAGGTCTGAAAGGCGAACAG (FAM)AGGAGAAATGCCTGACGAAGCTCTCCA (TAMRA)	Suzuki K, et al[14]
28S rRNA	GGCGAAGCCAGAGGAAACT GACGACCGATTTGCACGTC (FAM) AGGACCGCTACGGACCTCCACCA (TAMRA)	
IFN-ɣ	AAACAACCTTCCTGATGGCGT CTGGATTCTCAAGTCGTTCATCG (FAM) TGAAAGATATCATGGACCTGGCCAAGCTC (TAMRA)	Markowski-Grimsrud, et al[15]

mL in phosphate-buffered saline, PBS) from Sigma Chemical Company was washed with sterilized PBS, then coating with complement through the process of opsonization by incubation with species serum for 1h at 37°C, then centrifuged and resuspended in sterilized PBS. For the assay of phagocytosis, cells were incubated with zymosan particles for 1 h and overnight, at each time the supernatant over macrophage was collected and NO concentration was measured.[17] The test depends on nitrite being a stable oxidation product of NO, which correlates with the amount of NO present in the supernatant of macrophage. The amount of stable nitrite was determined by mixing the supernatant of macrophage with colorless Griess reagent which results in the formation of a purple complex. The degree of the color development was measured spectrophotometrically using an ELISA reader at 570 nm.

Evaluation of humoral immune response

ELISA test

The MG antibody test kit (BioChek, United States, Cat. No. CK114) was used to monitor the immune response to MG in the serum of SPF chickens after vaccination. This test was performed according to the manufacturer's instructions and the results were interpreted as: a sample with an antibody titer of 668 or greater was considered positive, while a lower titer indicated no antibody detection (negative).

The MS antibody test kit (BioChek, United States, Cat. No. CK115) was used to monitor immune response to MS after vaccination. This test was performed according to the manufacturer's instructions and the results were interpreted as: a sample with an antibody titer of 843 or greater was considered positive, while a lower titer indicated no antibody detection (negative).

Challenge test[7]

Twenty eight days after vaccination, each of the vaccinated chickens (25) and controls (10) were challenged by inoculation into abdominal air sacs, with 10^6 colony forming unit (CFU)/mL of the virulent strain of MG and 10^7 CFU/mL of the virulent strain of MS (which is sufficient to produce gross lesions of air saculitis with an arithmetic mean score of at least 3 in controls within 14 days after challenge). Challenged birds were observed daily for 14 days; succumbed birds were examined for air saculitis gross lesions. At the end of the observation period, all survival challenged birds were euthanized and air saculitis gross lesions were examined and scored as follow:

0: Clear, no lesions.

1: Slight cloudiness and thickening of the membranes with fibrinous exudates.

2: Moderate cloudiness and thickening of the membranes with fibrinous exudates.

3: Severe cloudiness and thickening of the membranes with fibrinous exudates.

4: Extensive and/or severe cloudiness and thickening of the membranes with fibrinous and casious exudates.

The mean scores air saculitis gross lesions in the challenged groups (vaccinated and control) were calculated; the percentage of protection was estimated as follows:

Protection (%) = mean score of air saculitis gross lesions in challenged control group – in challenged vaccinated group x 100 / mean score of air saculitis gross lesions in challenged control group.

The test was not valid unless the challenged control group recorded a mean mycoplasmosis air saculitis gross lesions score of at least 3. The vaccine was considered potent if the percentage of protection was not less than 70%.

Ethical approval

The Institutional Animal Care and Use Committee at the Central Laboratory for Evaluation of Veterinary Biologics acknowledged that the research manuscript was reviewed under its research authority and was deemed in compliance to the bioethical standards.

Statistical analysis

Data generated from immune responses were subjected to one-way analysis of variance (ANOVA). Variant means were separated post hoc using the least

significant difference (LSD) method; $p<0.05$ were accepted as significant.

Results

Cell mediated immune response

Lymphocyte proliferation assay

Lymphocyte proliferation response in heparinized blood of vaccinated and non-vaccinated groups was carried out on 0, 1st, 2nd, 3rd, 4th, 5th, 6th, and 7th DPV. From Figure 1, it was found that the level of lymphocyte proliferation in all vaccinated groups after stimulation was significantly higher than in the non-vaccinated group. In addition, this level increased gradually from the 1st DPV recording 1.576 and 1.394 and reached the optimum with a value 4.006 and 4.118 at 7th DPV for G1 and G2, respectively.

Cytokine mRNA gene expression of IL-6 and IFN-γ in spleen of chickens

The results of mRNA of IL-6 and IFN-γ in chicken's spleen were illustrated in Figures 2 and 3. Data revealed that the level of IL-6 decreased along the course of the experiment, while expression for IFN-γ was persistently up regulated along the course of experiment.

Nitric oxide level

The data illustrated in Figure 4 showed that the NO level at 0, 1st, 2nd, 3rd, 4th, 5th, and 7th DPV for groups G1, G2 and G3. These results showed that the level of NO increased gradually from 1st until to 7th DPV. In addition, it was found that the level of NO in vaccinated SPF chickens was significantly higher than in the non-vaccinated group.

Humoral immune response

ELISA test

The data illustrated in Table 2 reveal that antibody titers against MG and MS at 1st WPV were 2,219.97 and 2,095.75, respectively. After that, the antibody titers for both vaccines gradually increased to reach a peak value of 4,391.09 and 3,507.23 at 4th WPV, respectively. A high significance was noticed for vaccinated SPF chickens respect to control group along the experiment.

Challenge test

The percentage of protection for vaccinated SPF chickens with MG vaccine is shown in Table 3. The group inoculated with MS vaccine had a higher protection percentage at 4th WPV.

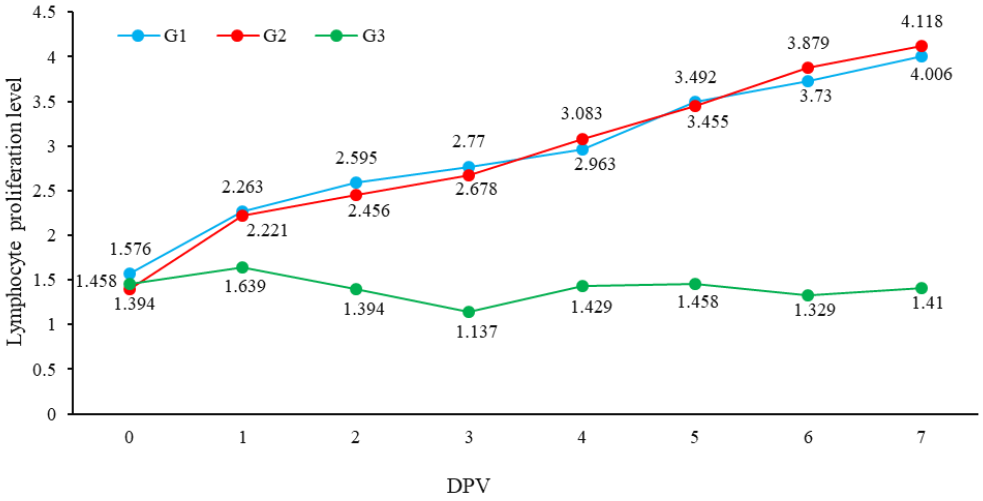

Fig. 1. Absorbance for the lymphocyte proliferation assay using XTT cell proliferation assay by spectrophotometer. G1: group of SPF chickens vaccinated with MG vaccine**.** G2: group of SPF chickens vaccinated with MS vaccine. G3: unvaccinated group (-ve control). DPV: days post vaccination.

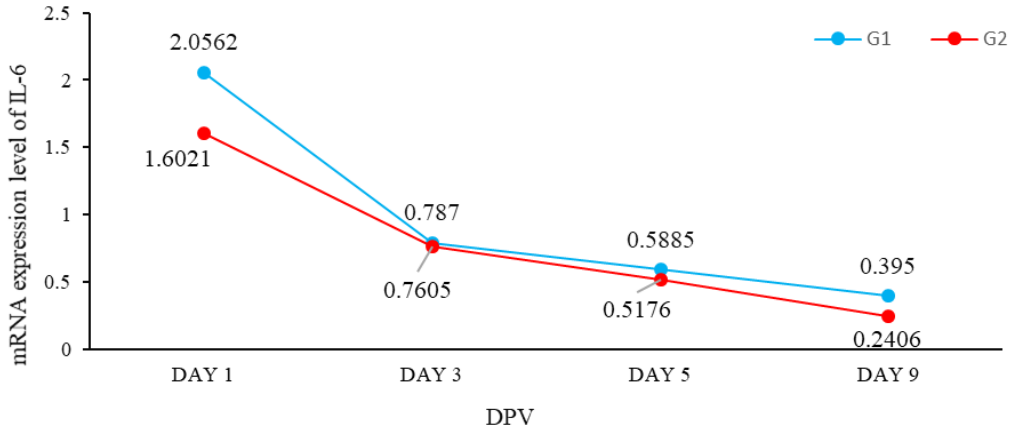

Fig. 2. Measurement of IL6 gene expression using real time PCR. G1: group of SPF chickens vaccinated with MG vaccine. G2: group of SPF chickens vaccinated with MS vaccine. DPV: days post vaccination.

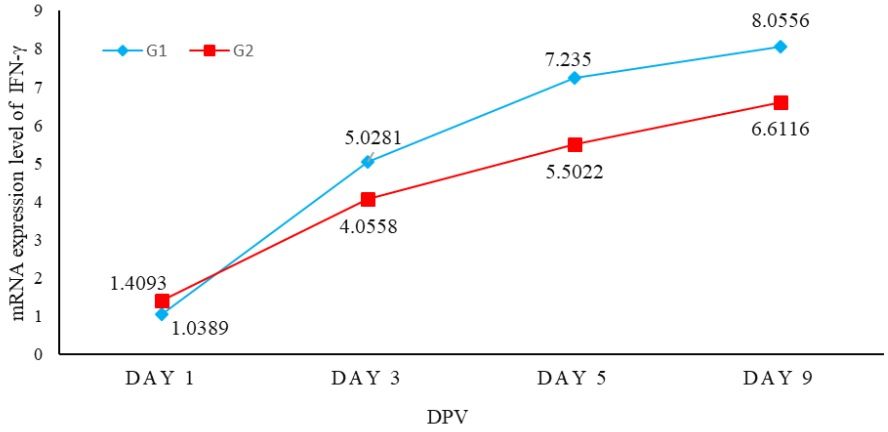

Fig. 3. Measurement of IFNγ gene expression using real time PCR. G1: group of SPF chickens vaccinated with MG vaccine**.** G2: group of SPF chickens vaccinated with MS vaccine. DPV: days post vaccination.

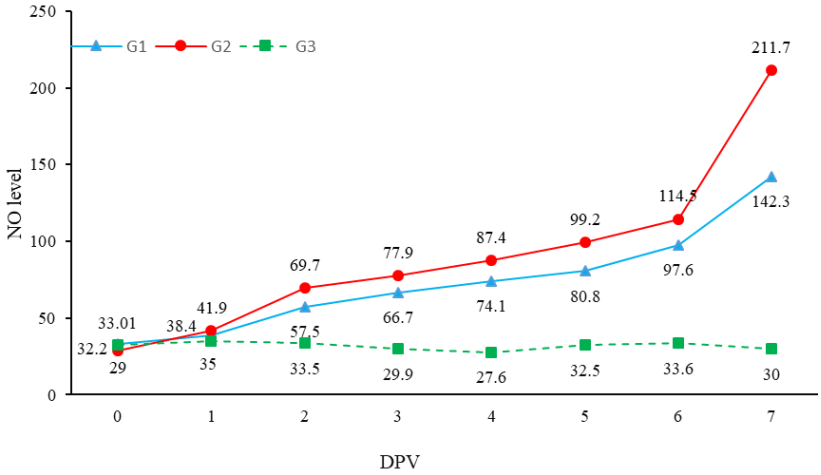

Fig. 4. Estimation of nitric oxide concentration in the supernatant of macrophage. G1: group of SPF chickens vaccinated with MG vaccine. G2: group of SPF chickens vaccinated with MS vaccine. G3: unvaccinated group (-ve control). NO: nitric oxide. DPV: days post vaccination.

Table 2. Antibody titers against MG and MS.

Vaccine WPV	**Mean antibody titer against MG**		**Mean antibody titers against MS**	
	Vaccinated	**Control**	**Vaccinated**	**Control**
1	2,219.97	169	2,095.75	121.5
2	2,933.73	175	2,264.27	95.27
3	3,620.92	188.79	2,793.89	87.16
4	4,391.09	168.9	3,407.23	79.43

WPV: week post vaccination.

Table 3. Protection (%) of vaccinated MG and MS against virulent MG and MS isolates, respectively.

Vaccine	**Challenge isolate**	**No of chickens with lesion**	**No of dead chickens**	**Protection (%)**
MG	MG	3/25	2/25	88
MS	MS	2/25	2/25	92

Discussion

Avian mycoplasmosis is a worldwide disease caused by bacteria in the genus mycoplasma that affects several bird species.[1] They are vertically and horizontally transmitted and clinical signs differ according to mycoplasma species, strain, and infected bird species.[1,2]

Vaccination is an important tool to limit the disease incidence. Live vaccines have a significant role in the control of mycoplasma infections.[6] Therefore, this study was aimed to evaluate the cell mediated and humoral immune response of two commercial live mycoplasma vaccines (MS-H and MG ts-11), moreover, to assess vaccine effectiveness in SPF chickens. For this purpose, 150 SPF chickens were clustered into three groups (50 SPF/group): MG ts-11vaccine, MS -H vaccine and control non vaccinated group. For identifying the cell-mediated immune response following vaccination, heparinized blood samples were collected at 0, 1st, 2nd , 3rd, 4th 5th, 6^{th,} and 7th days to perform lymphocyte proliferation assay and determine NO level in supernatant of macrophages. In addition, spleen tissues of the same groups were examined at 1st, 3rd, 5^{th,} and 9th DPV to quantify the expression of the genes responsible for IFNγ and IL-6 production using qRT-PCR. The proliferative ability of lymphocytes in each vaccine immunization group was measured using the XTT cell proliferation assay kit. The lymphpoliferative level was optimal at 7th DPV for both groups of vaccinated chickens and was significantly higher compared to the non-vaccinated group. No obvious cell proliferation was detected in the non-vaccinated group. These results agreed with those of other authors[18] that showed mycoplasma vaccination induced a strong proliferative response after a short period of vaccination.

The mRNA expression level of IL-6 and IFNγ determined using qRT-PCR revealed that the expression level of IL-6 gradually decreased from 1st until 9th DPV, which is consistent with another research[18] where the level of IL6 gradually decreased after MG vaccination. Also, IL-6 is a multifunction cytokine that is necessary for T cell differentiation and is involved in antibody formation as a B-cell differentiation.[8]

The level of IFNγ expression was increased along the experiment which is in agreement with a study[7] that estimated the level of IFNγ gene was significantly up-regulated throughout the experiment. Therefore, the IFNγ is known to play an important immunoregulatory role through its ability to activate macrophages and enhance MHC class II antigen expression on a variety of cell types, moreover, it can protect from infection despite low antibody titer.[19]

Another interesting result was that NO level in the macrophage supernatant of the two vaccinated groups increased gradually from 1st DPV until reach the maximum level at 7th DPV, with a high significant level respect to the control group. NO is a free radical gaseous molecule that is reported to be a mediator of a variety of vital physiological functions including phagocytosis and important antimicrobial and cytotoxic functions in some animals.[20]

The evaluation of the humoral immune response was assessed using an ELISA test. Collected sera of vaccinated chickens with the live mycoplasma vaccines ts-11 (against MG) and MS-H (against MS) were tested for antibodies using the MG indirect antibody test kit and the MS antibody test kit. The analysis of sera from mycoplasma-vaccinated groups revealed high significant differences respect to non-vaccinated group throughout the experimental interval. ELISA results showed that positive antibody titers against MG and MS appeared significantly from the 1st WPV and the level of antibodies gradually increased to reach the optimum at 4th WPV. These results agreed with other study[21] which reported that the live MS vaccine (MS-H) diffused extensively through the poultry population, as evidenced by the wide serological response (over 80% of positive samples in rapid serum agglutination test and 85% in ELISA) with high serological titers at the endpoint of the experiment. In parallel, the challenge test was applied to vaccinated and control groups, using a homologous local virulent strain of MG and MS. The obtained results showed the two live vaccines induced satisfactory protection level according to the manual for vaccine evaluation of the World Organization for Animal Health (WOAH),[6] stating that an effective mycoplasma live poultry vaccine should protect at least 70% of vaccinated chickens from death; these results agreed with others[19] which stated that vaccines based on MG adhesion and phase variation proteins are the most suitable candidates to prevent and control MG infection and to sustain better health, welfare, and production of poultry.

Conclusion

The two live commercial mycoplasma vaccines (MG ts-11 and MS -H) induced high lymphpoliferative activity, NO and IFNγ with a significant level along the experiment, while the level of IL6 gradually decreased from 1st to 9th DPV with a significant level respect to control group. The two live vaccines exhibited high seroconversion level at 4th WPV with satisfactory protection level after challenge using virulent (MG and MS) strains. Finally, the high protective efficacy of the two live mycoplasma vaccines evaluated could be attributed to the role of cell-mediated immunity and immunomodulatory cytokines.

Conflict of interest

The authors declare that there is no conflict of interest.

Author's contributions

Safwa Zaghloul-Mohamed: conducted the experiment and drafted the manuscript, designed and followed up the experiment and critically reviewed the manuscript.

Samir A. Nassif: conducted the experiments and drafted the manuscript, designed and followed up the experiment and critically reviewed the manuscript.

Randa E. El-Naggar: designed and followed up the experiment and critically reviewed the manuscript, participated in designing and followed up the practical work.

Ghada M. Elsadek: designed and followed up the experiment and critically reviewed the manuscript, participated in designing and followed up the practical work.

Elsayed I. Salim: designed and followed up the experiment and critically reviewed the manuscript.

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

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Evaluación de la eficacia y de la respuesta inmune celular y humoral inducida por dos vacunas contra micoplasma, en pollos libres de patógenos específicos

Resumen

Se estudió la respuesta inmune mediada por células y la humoral después de la vacunación con vacunas vivas contra micoplasma. Para ello se inocularon 100 pollos libres de patógenos específicos con dos diferentes vacunas vivas de micoplasma: *Mycoplasma gallisepticum* (ts-11) y *Mycoplasma synoviae* (H); otros 50 pollos se mantuvieron como control (pollos no vacunados). Para evaluar la respuesta inmune mediada por células, se obtuvieron leucocitos de sangre periférica de los grupos de pollos vacunados y controles y se determinó la respuesta proliferativa de los linfocitos y el nivel de óxido nítrico. Se evaluaron los perfiles de expresión de las citocinas inmunomoduladoras provocadas por la vacuna y se determinó el nivel de expresión del ARNm de la interleucina 6 y el interferón gamma en el bazo de los pollos libres de patógenos específicos vacunados. Paralelamente, los sueros recogidos de los pollos vacunados y controles se evaluaron mediante un ensayo inmunoenzimático para determinar la respuesta inmune humoral. En la 4ª semana posvacunación se aplicó a todos los pollos vacunados y controles una prueba de provocación contra cepas virulentas de *Mycoplasma gallisepticum* y *Mycoplasma synoviae*. Los resultados confirman la presencia de linfoproliferación con producción de interferón y óxido nítrico *in vitro* en los dos grupos de pollos vacunados en comparación con el control negativo. Además, las vacunas ensayadas indujeron un alto nivel de seroconversión con una protección satisfactoria (%) a la 4ª semana posvacunación. Se concluyó que las dos vacunas vivas contra micoplasma pueden proteger a los pollos vacunados contra las infecciones por *Mycoplasma gallisepticum* y *Mycoplasma synoviae*, sugiriendo un papel significativo para la inmunidad celular.

Palabras clave: inmunidad celular; inmunidad humoral; interleucina-6; infecciones por Mycoplasma; vacunas.

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