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Trials for Preparation and Evaluation of Combined Inactivated *Mycoplasma Gallisepticum* and *Synoviae* Vaccine in Chicken and Turkey

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ABSTRACT

A large number of poultry farms around the world have been shut down during the last few years due to avian Mycoplasmosis, especially those where chickens and turkeys were raised. Therefore, the main goal of this study was to investigate the possibility of eradicating the disease by immunizing one-week-old chicks and turkey poults against *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) using an inactivated single dose or a combined vaccine. In this study, a total of 280 one-week-old Specific-Pathogen-Free chicks were divided into four groups to evaluate their immune response following vaccination. Chicks of group 1 (n=60) were given inactivated MG vaccine, those of group 2 (n=60) were given inactivated MS vaccine. Chicks of group 3 (n=120) received combined inactivated MG+MS vaccine, while birds of group 4 (n=40) served as control. After three weeks, the chicks were administered with the booster dose of the respective vaccine. Similarly, 60 turkey poults of one week in age were divided into two groups: Poults of group 1 (n=50) received combined inactivated MG+MS vaccine, while those of group 2 (n=10) were kept as control. The booster dose of vaccine in group 1 birds was given after 4 weeks. The immune responses of vaccinated chicks and turkey poults were measured by HI test, ELISA and challenge test. The results of this study revealed that the combined inactivated MG+MS vaccine adjuvanted with Montanite ISA70 was more effective against *Mycoplasma synoviae* and *Mycoplasma gallisepticum* in chickens and turkey poults than the individual inactivated vaccine and there was significant difference in the group of turkey poult vaccinated with combined inactivated MG-MS in ELISA test.

Key words: ELISA, HI Test, Inactivated Vaccines, *Mycoplasmosis.*

INTRODUCTION

Mycoplasma species are extremely small prokaryotes that range in size from 300 to 800 nm. They lack the cell wall and are surrounded by a triple-layered plasma membrane. They are slow-growing, relatively fastidious organisms that typically require a temperature from 35 to 37°C for growth (Yadav et al. 2021; El-Naggar et al. 2022; Chaidez-Ibarra et al. 2022). There are four famous species of the 23 known avian mycoplasma species which show special affinity for poultry. *Mycoplasma gallisepticum* (MG) causes infections in chickens, turkeys, duck, and wide variety of free-living poultry. It causes catarrhal rhinitis, tracheitis, pneumonia, edema of the air sac walls and sinusitis in different species of poultry (Hernández 2014).

However, *Mycoplasma synoviae* (MS) infection is typically linked to poor growth and severe deterioration of carcasses, a decrease in egg production, and the creation of eggshell apex abnormalities. MS infection most states require as an asymptomatic upper respiratory illness. Synovitis, the main MS disease, affects the synovial tendon sheath and joint synovium, resulting in pale combs, lameness, stunted growth, and swelling around the joints in hens (Galluzzo et al. 2022).

Poor management system managed by veterinarian and workers, accompanied by poor hygienic conditions, leads to fastidious respiratory disease of poultry caused by

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pathogenic *Mycoplasma*, mainly *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). It is quite difficult to eradicate avian Mycoplasmosis from the infected farms. Both the MS and MG strains of avian Mycoplasma vary in their mechanisms of virulence and infectivity (OIE 2018).

Mycoplasma gallisepticum and *Mycoplasma synoviae* are etiological agents for complex respiratory disturbances in poultry. Additionally, MS produces articular problems, while MG infection can reduce egg production (Eissa 2019; Qadir et al. 2021). The MS is a pathogen which is also associated with extra respiratory tract infection (Behboudi 2022; Bastamy et al. 2022).

Clinically and financially, MS has always been viewed as a secondary pathogen [8]. However, due to the discovery of novel, particularly virulent strains, MS surveillance and management have become more crucial in the commercial poultry industry over the past 20 years (Kursa et al. 2019).

Before colonizing in the respiratory mucosa of the host, *M. gallisepticum* first attaches to its target epithelial cells. A primary cytadhesin (Gap A) and cytadhesin-related molecules (Crm), such as CrmA, are involved in this interaction. It is believed that the degree of immunity produced by vaccines depends on the viability of attachment of microorganisms to respiratory epithelial cells and their subsequent colonization (Kulappu et al. 2021).

Numerous investigations have revealed the existence of MG and MS infections in birds other than chickens and turkeys, such as various game birds like pheasants, partridges, and quails, wild birds like sparrows, house and gold finches, pigeons, crows, and flamingos, and waterfowl like ducks and geese (Anneke et al. 2022).

There is a specific strain, *Mycoplasma meleagridis* which affects turkeys, causing air-sacculitis (Chin 2013). There is another strain, *Mycoplasma iowae* which can infect turkeys and poor-quality poults; it has six different virulent serovars (Bradbury and Raviv 2013).

Poultry farmers work hard to decrease the losses faced by the poultry industry due to mycoplasma, particularly in layers and breeding flocks, by employing a variety of vaccines including outer membrane vaccines, killed vaccines, and live vaccines. These vaccines are able to reduce the severity of the respiratory illnesses, maintain continuous egg production and decrease horizontal and vertical transmission of the infection (Butcher 2002).

In previous studies, different types of vaccines such as live-attenuated vaccines, killed vaccines, bacterin-based, or subunits have been used (Hussein et al. 2007; Rabie and Amin Girh 2020; Kulappu et al. 2021). These vaccinations worked by enhancing and activating the immune system by adding oil adjuvant to the dead pathogens or bacteria (Ley 2003). According to Ishfaq et al. (2020), use of inactivated bacterins is safer than using live vaccines to prevent chicken respiratory lesions and reduce vertical or horizontal transmission of the disease as the spreading of both MG and MS is due to both horizontal and vertical transmission. Both the strain involved and the stage of infection (from 3–4 weeks in the acute phase to decline in the chronic phase) may have an impact on the transmission rate in eggs (Derksen et al. 2018). These mycoplasmas can also spread horizontally by direct or indirect contact, aerosol transfer, introduction of contaminated objects, or contaminated personnel (Viviana et al. 2020) Numerous serological assays, including HI, ELISA and rapid serum agglutination tests, are used most frequently (OIE 2018) to estimate MG or MS antibodies.

The present study was planned to prepare and evaluate an effective protective inactivated vaccine against *Mycoplasma synoviae* and *Mycoplasma gallisepticum* infection in chickens and turkey poults.

MATERIALS AND METHODS

Ethical Approval

The committee of animal care at the Central Laboratory for Evaluation of Veterinary Biologics has approved to conduct this work on experimental chickens and turkey poults.

M. synoviae and *M. gallisepticum*

The *M. synoviae* and *M. gallisepticum* strains were obtained from the Animal Health Research Institute, Mycoplasma Department, Dokki, Giza and used in the preparation of vaccines and the challenge test.

Laboratory Animals

Embryonated Chicken Eggs (ECE)

Thirty specific pathogen free (SPF) eggs (6 days old) were used in the propagation of *M. synoviae* and *M. gallisepticum* via yolk sac to increase the virulence for best results for vaccine preparation and challenge test.

Chickens and Turkey Poults

A total of 310 specific-pathogen-free (SPF) one-day-old chicks were purchased from SPF farm Kom Oshim, Fayoum, Egypt for this study. Similarly, 70, one-day-old turkey poults vaccinated against Newcastle, *E. coli* and Marek's disease were obtained from Faculty of Agriculture, Cairo University. These chickens and turkey poults were isolated and maintained in negative pressure specific isolators at the animal husbandry facilities of the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) under full supervision of researchers and workers.

Preparation of Separate Inactivated Oil Emulsion *M. gallisepticum* and *M. synoviae* Vaccines

The *M. gallisepticum* seed culture was inoculated into a starter culture flask (250 ml of Frey's medium, pH 7.8). Fresh medium was inoculated with 24 hours broth culture equivalent to 10% of the volume of medium used. Following 48 hours incubation at 37°C in carbon dioxide incubator, the mycoplasma cells were harvested and washed using PBS (pH 7.2) by centrifugation at 12,000 rpm for 30 minutes. After three successive washings, a final suspension of antigen was prepared to contain 1% packed cell volume (PCV) in PBS in the final product. The antigen batch was inactivated with 0.5% formalin with frequent agitation for 24 hours incubation at 37°C. The Montanide ISA70 oil adjuvant was added to batch of inactivated mycoplasma vaccine at 1:1 ratio. Finally, the thiomersal was added at a final concentration of 0.01%. The same protocol was adopted for the preparation of *M. synoviae* vaccine (Yoder 1979).

Preparation of Combined Inactivated Oil Emulsion Vaccine against *M. gallisepticum* and *M. Synoviae*

Equal parts (V/V) of the inactivated broth of MG and MS strains were mixed using a magnetic stirrer. The concentration of the aforementioned suspension was changed to contain $3x10^{10}$ CFU per dosage (5% PCV) of MG and MS in accordance with Yoder (1979). Then, equal amounts of aforementioned culture and Montanide ISA70 oil were mixed thoroughly at 50/50 ratio using a magnetic stirrer at approximately 300rpm for 15min (water-in-oil emulsions). Finally, the thiomersal was added at a final concentration of 0.01%.

Experimental Design

For Chickens

Two hundred and eighty SPF chicks of one week age were classified into four groups as follows:

Group 1: Sixty SPF chicks were vaccinated by MG inactivated vaccine. Group 2: Sixty SPF chicks were vaccinated by MS inactivated vaccine. Group 3: One hundred and twenty SPF chicks were vaccinated by combined MG and MS inactivated vaccine. In each group, the respective vaccine adjuvanted with Montanide ISA70 was given at a dose of 0.5mL S/C in upper dorsal part of neck ($3x10^{10}$ CFU). Group 4: Forty SPF chicks were injected with 0.5mL S/C normal saline and used as the control group.

Chicks in groups 1, 2 and 3 were boostered with the same vaccine through the same route and dose 3 weeks after first immunization. Serum samples were obtained regularly before immunization, weekly after each vaccination and post challenge for three weeks (once/week), then pooled and stored at -20°C till used for following up the induced antibodies.

For Turkey Poults

Sixty turkey poults of one week age were divided into two groups as follows:

Group 1: Fifty turkey poults were vaccinated with combined MG and MS inactivated vaccine adjuvanted with Montanide ISA70 at a dose of 0.5mL S/C in upper dorsal part of neck ($3x10^{10}$ CFU). Group 2: Ten turkey poults were injected with 0.5mL S/C normal saline and left as the control group.

Turkey poults in group 1 were boosted with the same vaccine via the same route and dose 4 weeks after first immunization. Serum samples were obtained regularly before immunization, weekly after each vaccination and post challenge, then pooled and stored at -20°C till used for following up the induced antibodies.

Quality Control of the Prepared Vaccines

The three prepared vaccines were tested for purity, sterility, and safety according to OIE (2018).

Purity and Sterility Tests

In accordance with OIE (2018), the prepared vaccines were tested for confirmation that the vaccines were free from any bacterial or fungal contamination. So, the tested vaccines were inoculated on thioglycolate broth and incubated at 37°C for 48-72hrs and on soya casein digestine agar at 25°C for 14 days.

Safety Tests

SPF Chicks Inoculation Test

A total of 30 SPF chicks aged one week were inoculated subcutaneously with 1.0mL (double field dose) per bird of the *M. gallisepticum* vaccine, *M. Synoviae* vaccine and combined vaccine of *M. gallisepticum* and *M. synoviae* (10 chicks for each vaccine). Inoculated chicks were kept under observation for 14 days to ensure the safety of the prepared vaccines (OIE 2018).

Turkeys Inoculation Test

A total of 10 turkey poults aged one week were inoculated subcutaneously with 1.0mL (double field dose) of combined MG and MS vaccine and the inoculated turkey poults were kept under observation for 14 days to ensure the safety of the prepared vaccine (OIE 2018).

Evaluation of Immune Response of Prepared Vaccines

Rapid Serum Agglutination Test

The Rapid Serum Agglutination test was carried out at room temperature (20-25˚C) within 72hrs of serum collection, the reagents were also brought to the room temperature and to reduce non-specific reactions, centrifugation was done. A drop containing 0.02mL of serum was put onto a clean glass slide from each serum sample, and then 0.02mL of stained antigen of *Mycoplasma gallisepticum* (Lot No: 01143 *Mycoplasma gallisepticum* Ag produced by Salsbury laboratories) was added. The antigen bottle was shacked for 2min before use. Detailed protocol for the Rapid Serum Agglutination test has been described elsewhere (Avakian et al. 1988).

Hemagglutination Inhibition Test

Hemagglutination Inhibition Test was performed on the serum samples collected after vaccination by using Mycoplasma strains according to the protocol described earlier (Senterifit 1983)**.**

Elisa

Kits ID Vet ® *Mycoplasma gallisepticum* 0416 and ID Vet ® *Mycoplasma synoviae* 0416 were used to measure immune response against MG and MS, respectively according to protocol provided by the manufacturer of kits.

Challenge Test

Challenge test against MG and MS in the vaccinated and challenged groups was performed. For this purpose, 0.1mL of $3.8x10^{6}$ CFU/mL of MG or MS strain was inoculated via intra-air sac route (OIE 2018). The protection percentage was calculated as: number of challenged birds with no deaths/total number of challenged birds x100.

Statistical Analysis

Data obtained in this study were analyzed using Statistical packages for social science (SPSS) software (version 26.0 for windows 10) (IBM Corp., 2019). Comparison of means using Independent T-test was carried out to express HI and ELISA test for inactivated *Mycoplasma synovae*, inactivated *Mycoplasma gallisepticum* and inactivated combined MG-MS vaccine.

RESULTS

Quality of the Vaccines

The experimental vaccines were found to be free from any bacterial, fungal and mycoplasma contaminant and safe for use in chickens and turkey poults.

The Immune Response of Vaccinated Birds

Rapid Serum Agglutination Test

In less than two minutes, all pre-vaccinated blood samples (Fig. 1) and tested serum samples from the vaccinated chickens, turkey poults and control groups gave positive agglutination.

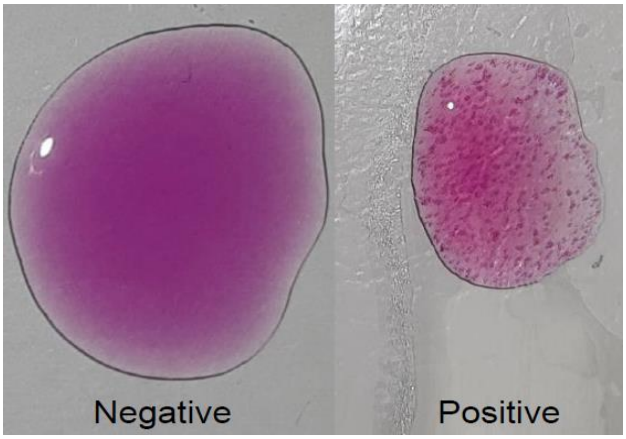

Fig. 1: Photograph showing the result of Rapid Serum Agglutination test.

Hemagglutination Inhibition Test

The immune response of vaccinated chickens against MG was determined by the HI Test (Table 1), which revealed that antibody titers against MG increased in chickens of group 3 (given the combined inactivated MG-MS vaccine) in the second and third week after the first vaccination and reached 256, while antibody titers against MG in group 1 reached 128 at 3rd week after first vaccination and reached 512 after three weeks from the second vaccination, and there were noticeable decrease in antibody titers after that.

As shown in Table 2, the inactivated combined MG-MS vaccine in group 3 resulted in an improvement in antibody titers against MS in the second and third weeks following the initial vaccination, reaching 128, while the antibody titer for the single dose reached 32 and the antibody titer for the double dose reached 512 after three weeks of booster vaccination. The obtained results of HI test illustrated in Tables 1 and 2 were analyzed statistically to compare group 1 and group 3 for MG and also compare group 2 and group 3 for MS. This analysis showed significant differences ($P<0.05$) between these groups.

As shown in Table 3, the immune response of immunized turkey poults against inactive MG increased in the second and third week following the first vaccination, reaching 32 and 128 respectively, and increased to 1024 after three weeks following the second immunization, whereas in the control group, it remained zero throughout the study. Fig. 2 and 3 depict the clean air sac of immunized chickens and the muddy air sac of unimmunized chicks, respectively.

Table 1: Antibody titer of vaccinated chickens against *M. gallisepticum* by HI Test

	Geometric mean antibody titer								
Group	Pre-vaccination	Weeks post 1st vaccination			Weeks without boostering (Single dose)			Weeks post boostering (Double dose)		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Group 1	0	32	64	128	128	64	32	128	256	512
Group 3	0	32	128	256	256	128	64	256	512	1024
Group 4	0	0	0	0	0	0	0	0	0	0

There was not a significant difference between group 1, group 3 and group 4 (t=1.217, P=0.239). Group 1: Chickens vaccinated with inactivated MG vaccine (Single dose - Double dose). Group 3: Chickens vaccinated with combined inactivated MG-MS vaccine (Single dose - Double dose). Group 4: Control group.

Table 2: Antibody titer of vaccinated chickens against *M. synoviae* by HI Test

	Geometric mean antibody titer								
Groups	Pre-vaccination	Weeks post 1st vaccination			Weeks without boostering (Single dose)			Weeks post boostering (Double dose)		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Group 2	0	16	32	64	64	32	16	64	128	256
Group 3	0	32	64	128	128	64	32	128	256	512
Group 4	0	0	0	0	0	0	0	0	0	0

There was not a significant difference between group 2, group 3 and group 4 (t=1.255, P=0.226). Group 2: Chickens vaccinated with inactivated MS vaccine (Single dose - Double dose). Group 3: Chickens vaccinated with combined inactivated MG-MS vaccine (Single dose - Double dose). Group 4: Control group.

Table 3: Antibody titer of vaccinated turkey poults against *M. gallisepticum* by HI Test

Groups	Pre-vaccination	Weeks post 1st vaccination			Weeks post boostering		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week
Group 1	0	16	32	128	256	512	1024
Group 2	0	0	0	0	0	0	0

There was not a significant difference between group 1, and group 2 (t=1.987, P=0.07). Group 1: Turkey poults vaccinated with combined inactivated MG-MS vaccine Group 2: Control group.

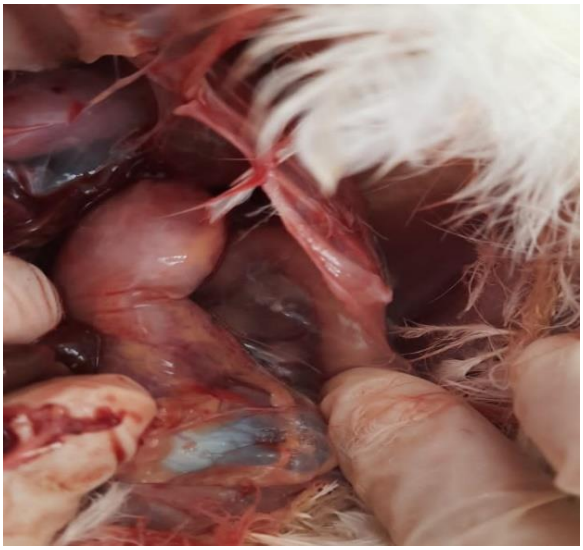

Fig. 2: Photograph showing clear air sac of vaccinated chickens.

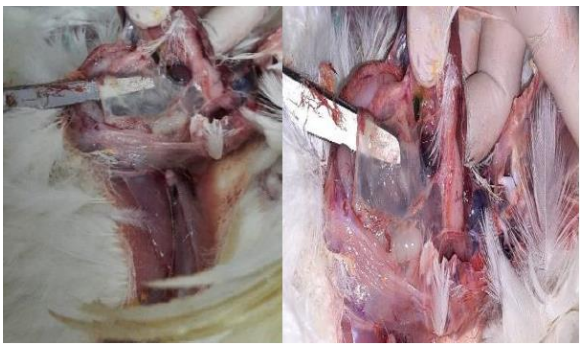

Fig. 3: Photograph showing turbid air sac of non-vaccinated chickens.

The immune response of immunized turkey poults against MS is shown in Table 4. The antibody titer against MS in the inactivated combined MG-MS vaccine in group 1increased in the second and third week following the first vaccination, reaching 32 and 64 respectively, and increased to 512 after three weeks from the second vaccination. However, the titer remained 0 in the control group.

ELISA

The results of antibody titers in chickens measured by ELISA (Table 5) showed increase in antibody titers against MG in the inactivated combined MG-MS vaccine in group 3 in the second and third weeks after first vaccination and reached 2638 and 3165, respectively; while in single dose it reached 3111 and 2586 respectively and reached 9235 after three weeks from second vaccination. However, for inactivated MG vaccine in group 1 it reached 7260 after three weeks from second vaccination. There were marked decreases in antibody titers after six weeks from first vaccination for inactivated combined MG-MS vaccine in group 3 and inactivated MG vaccine in group 1, reaching 2586 and 1604, respectively.

The data of ELISA presented in Table 5 were statistically analyzed to compare group 1 and group 3 for antibody titer against MG. This analysis showed significant difference in antibody titer between the two groups ($P<0.05$).

In the present study, the antibody titer against MS also increased for the inactivated combined MG-MS vaccine (group 3) in the second and third week after first vaccination when it reached 1249 and 3079, respectively (Table 6). However, in single dose it reached 2809 and 2286 at 2nd and 3rd week, respectively and reached 4797 after three weeks from second vaccination. For inactivated MS vaccine in group 2, the titer reached 3353 three weeks after second vaccination and there were marked decreases in antibody titers after six weeks from first vaccination in inactivated combined MG-MS vaccine (reaching 2286) and inactivated MS vaccine (reaching 1784).

The presented in Table 6 were statistically analyzed to compare antibody titer between group 2 and group 3 for MS. This analysis revealed significant difference between group 2 and group 3 ($P<0.05$).

The immune response against MG in turkey poults is shown in Table 7. The antibody titers against MG for the combined MG-MS vaccine increased in the second and third week after first vaccination, reaching 1697 and 1985, respectively; and increased to 7260 three weeks after second vaccination, when it was 120 in control group. Similarly, the antibody titer against MS for the inactivated

Table 4: Antibody titer of vaccinated turkey poults against *M. synoviae* by HI Test

Groups	Pre-vaccination	Weeks post 1st vaccination			Weeks post boostering		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week
Group 1	0	16	32	64	128	256	512
Group 2	0	0	0	0	0	0	0

There was not a significant difference between group 1, and group 2 ($t=2.065$, $P=0.061$). Group 1: Turkey poults vaccinated with combined inactivated MG-MS vaccine. Group 2: Control group.

Table 5: Antibody titer of vaccinated chickens against *M. gallisepticum* by ELISA

		Geometric mean antibody titer								
Groups	Pre-vaccination	Weeks post 1st vaccination			Weeks without boostering (Single dose)			Weeks post boostering (Double dose)		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Group 1	122	230	1792	2033	2586	2038	1604	3219	4799	7260
Group 3	122	336	2638	3165	3656	3111	2586	4799	6625	9235
Group 4	122	100	188	187	133	139	123	130	135	139

There was not a significant difference between group 1, group 3 and group 4 ($t=0.965$, $P=0.347$). Group 1: chickens vaccinated with inactivated MG vaccine (Single dose - Double dose). Group 3: chickens vaccinated with combined inactivated MG-MS vaccine (Single dose - Double dose). Group 4: control group.

Table 6: Antibody titer of vaccinated chickens against *M. synoviae* by ELISA

Groups	Pre-vaccination	Geometric mean antibody titer								
		Weeks post 1st vaccination			Weeks without boostering (Single dose)			Weeks post boostering (Double dose)		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week	1st week	2nd week	3rd week
Group 2	115	167	887	2033	2286	2033	1784	2545	2809	3353
Group 3	115	214	1249	3079	3353	2809	2286	3633	4206	4797
Group 4	115	112	178	177	107	124	123	130	135	139

There was not a significant difference between group 2, group 3 and group 4 (t=1.262, P=0.223). Group 2: chickens vaccinated with inactivated MS vaccine (Single dose - Double dose). Group 3: chickens vaccinated with combined inactivated MG-MS vaccine (Single dose - Double dose). Group 4: control group.

Table 7: Antibody titer of vaccinated turkey poults against *M. gallisepticum* by ELISA

Groups	Pre-vaccination	Weeks post 1st vaccination			Weeks post boostering		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week
Group 1*	100	255	1697	1985	3656	6002	7260
Group 2	100	113	110	109	183	171	120

There was a significant difference between group 1, and group 2 (t=2.729, P=0.018). Group 1: Turkey poults vaccinated with combined inactivated MG-MS vaccine. Group 2: control group.

Table 8: Antibody titer of vaccinated turkey poults against *M. synoviae* by ELISA

Groups	Pre-vaccination	Weeks post 1st vaccination			Weeks post boostering		
		1st week	2nd week	3rd week	1st week	2nd week	3rd week
Group 1*	100	221	943	2033	2286	2809	3353
Group 2	100	120	112	138	111	137	170

There was a significant difference between group 1, and group 2 (t=3.221, P=0.007). Group 1: Turkey poults vaccinated with combined inactivated MG-MS vaccine. Group 2: control group.

combined MG-MS vaccine increased in the second and third week after first vaccination, reaching 943 and 2033 respectively, and increased to 3353 three weeks after second vaccination, when it was 170 in control birds (Table 8).

As shown in Tables 9 and 10, after the challenge test against MG and MS strains, the protection percentages in chickens vaccinated with single dose in group 1 was 75%, while in group 2 it was 70% and in group 3 values were 80 and 75% for MS and MG, while in group 4 these were 0 and 10% for MG and MS, respectively.

Table 9: Air sac lesion and protection percentage in chickens vaccinated with single dose and then challenged with virulent *M. gallisepticum* and *M. synoviae* strain

Groups	Strains	No. of Chickens	Air sac Lesion	No. of Deaths	Protection Percentage
Group 1	MG	20	6	5	75
Group 2	MS	20	7	6	70
Group 3	MS	20	5	4	80
Group 3	MG	20	5	5	75
Group 4	MG	10	10	10	0
Group 4	MS	10	10	9	10

Table 10: Air sac lesion and protection percentage in chickens vaccinated with double dose of vaccine and challenged with virulent *M. gallisepticum* and *M. synoviae* strain

Groups	strains	No. of chickens	Air sac lesion	No. of deaths	Protection percentage
Group 1	MG	20	6	5	75
Group 2	MS	20	6	5	75
Group 3	MG	20	3	2	90
Group 3	MS	20	4	3	85
Group 4	MG	10	10	8	20
Group 4	MS	10	10	8	20

The protection percentages in chickens vaccinated with double dose in groups 1 and 2 were 75% each; in group 3 values were 90 and 85% for MG and MS respectively, while in control group the value was 20% for each strain. In turkey poults, after the challenge test against MG and MS strains, the protection percentage was 85% against MG and MS strains. In control group, it was only 10% against MG and 0% against MS (Table 11).

Table 11: Air sac lesion and protection percentage in turkeys vaccinated with double dose of combined inactivated MGMS vaccine challenged with *M. gallisepticum* and *M. synoviae* strains

Groups	Strains	No. of turkeys	Air sac lesion	No. of deaths	Protection percentage
Group 1	MG	20	5	3	85
Group 1	MS	20	3	3	85
Group 2	MG	10	10	9	10
Group 2	MS	10	10	10	0

DISCUSSION

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are the most virulent strains, causing avian mycoplasmosis in poultry with lack of a good management system in poultry farms. This adversely affects meat and egg production in poultry (Chaidez-Ibarra et al. 2022).

Mycoplasma synoviae may cause respiratory disease and synovitis or may result in a silent infection. Both MG and MS strains vary in their infectivity and virulence (Klose et al. 2022). The prevalence of MG and MS among commercial and rural laying hens in Italy was reported by Galluzzo et al. (2022). In the flocks under study, the prevalence of MG was 28.6% (commercial) and 40% (rural), and the prevalence of MS was 42.8% (commercial) and 44% (rural). For MG and MS, the overall prevalence at the animal level was 12.5 and 23.25%, respectively. Data provided indicate that MG is more common than MS in the farms under study. Additionally, these diseases were transmitted in urban and rural farms, highlighting the significance of monitoring

and controlling these illnesses and find best way to control this disease.

When given to healthy chickens via the upper respiratory tract, the F strain of MG has been the mostly used live vaccination strain, as little or no respiratory reactivity is noticed when this strain is used. However, respiratory symptoms and air-sacculitis may develop if the medication is administered through aerosol or if other respiratory disease agents like Newcastle disease or the infectious bronchitis virus are present (El-Naggar et al. 2022). The F strain of MG is a naturally occurring strain that affects hens with a mild to moderate severity, but it is virulent for turkeys. When birds are kept in close proximity to one another, the transmission of the avirulent strains ts-11 and 6/85 to unvaccinated birds either do not occur or the symptoms are very mild. Therefore, use of a live vaccine is not risk-free.

Mycoplasma gallisepticum and *Mycoplasma synoviae* recombinant vaccine were developed and tested by Eissa (2019), and the results showed that there was a significant difference between the vaccinated and non-vaccinated groups for serum and egg yolk ($P<0.05$). However, generally, the vaccinated groups had significantly higher antibody titers than the unvaccinated group in both serum and egg yolk, with no significant difference in the Geometric mean titers (GMTs) of the vaccinated groups. The outcomes showed that the recombinant vaccine was successful in preventing Mycoplasma infection in vaccinated birds. In recent studies (Condello et al. 2020; Klose et al. 2022) use of live attenuated vaccine using MG strain has been reported to be safe and gave effective protection. Kanci et al. (2018) evaluated the MG ts-304 vaccine as a live attenuated vaccination candidate for use in turkeys and found it to be a promising contender.

According to Ishfaq et al. (2020), a live MS-H vaccine against MS in turkeys inhibits field infections of MS. Live attenuated temperature sensitive vaccines of chickens are usually applied for the suppression of *Mycoplasma gallisepticum* infection in meat-type turkeys. The *Mycoplasma synoviae* MS-H vaccine has been found to be safe and effective in turkeys by Mohammadi et al. (2007). Oil based MG bacterin (killed Montanide ISA 70 *Mycoplasma gallisepticum* trial vaccine) protected infection from MG in Ethiopia (Legesse and Temesgen 2018). Additionally, Marouf et al. (2022) utilized inactivated pentavalent vaccination against mycoplasmosis and salmonellosis for hens. The manufactured vaccine was adjuvanted with Montanide ISA70 oil and demonstrated significant protective antibody titers against Salmonella and Mycoplasma with 100% efficiency.

To test the efficiency of the combination of inactivated MG-MS strains for totally inactivating mycoplasma strains, chicken and turkey poults were given either a single dosage or a double dose. For the purpose of preventing the spread of avian mycoplasmosis, numerous vaccine experiments are being conducted using live, subunit, and inactivated vaccines. It has been demonstrated that all of these vaccinations provide excellent protection against avian mycoplasmosis.

In this study, dual inactivated MG-MS vaccination in Group 3 chicks led to higher MG strain titers than a single vaccination in Group 1. A double dose furthermore revealed larger titers than a single dose. These outcomes are consistent with those reported by Fatma et al. (2015), who found that, in terms of HI test results and titer against MS strain, the second vaccination with inactivated immunostimulating polyvalent vaccine of M. gallisepticum and E. coli increased antibody titer more than the single vaccination. Additionally, the combined inactivated MG-MS vaccine produced a stronger immune response in turkey poults against both the MG and MS strains. These results are confirmed by those of Kanci et al. (2018), who discovered that MG ts-304 was safe even at a ten-fold overdose and could enter and remain in the trachea of turkey poults.

The combined inactivated MG-MS vaccination administered to chickens in group 3 in the current investigation produced greater titers than the single MS vaccine, it was revealed (group 2). Additionally, the immunological response from the double dosage vaccine was greater than that from the single dose. These results are in line with those of Fatma et al. (2015), who found that the inactivated polyvalent vaccine of MG and E. coli administered after a second vaccination increased antibody titer more than a single vaccination.

The inactivated immunostimulating polyvalent vaccine of MG and Pasteurella, following a second immunization, elicited higher antibody titer, according to Fatma (2018). The combined inactivated MG-MS vaccine resulted in higher antibody titers against MG and MS strains than in control birds, as shown by the antibody titers of vaccinated turkey poults against MG strain and against MS strain. All of these results are consistent with those previously reported (Eissa 2019; El-Naggar et al. 2022). They prepared and evaluated recombinant vaccine and provided a good level of protection. These findings are in line with those made by (Marouf et al. 2022), who discovered that inactivated pentavalent vaccine produced locally provides protection to birds and can be used as a powerful tool in combination with biosecurity measures to combat mycoplasmosis and salmonellosis in layer and breeder chicken farms in Egypt.

The protection rate of chickens that had received a single dose of the combined inactivated MG-MS vaccine (as shown in Table 9) and a double dose (as shown in Table 10) showed that the protection was 80% for the MS strain and 75% for the MG strain in chickens that had received a single dose, while it was 90% for the MG strain and 85% for the MS strain in chickens that had received a double dose. These findings are consistent with those previously published (Fatma et al. 2015), in which protection against Mycoplasma gallisepticum rose from 62% in chickens receiving a single dose of vaccination to 72% in those receiving a double dose. According to Fatma (2018), following the second vaccination, the combined inactivated immunostimulating polyvalent vaccine of MG and Pasteurella provided greater protection (up to 93%) than the individual MG vaccine (80%).

When challenged with M. gallisepticum and M. synoviae, turkey poults who had received a double dose of the combined inactivated MG-MS vaccination showed 85% protection against both strains, compared to 10% for the MG strain and 0% for the MS strain in the control group. The technique of vaccination with adjuvant ISA 71 VG followed by challenge test, according to Gong et al.

(2020), significantly increased antibody titers, decreased air sac tracheal lesions, and decreased MG and MS colonization compared to the other groups (P<0.05).

Conclusion

Based on the results of the present study, it can be concluded that the combined inactivated MG-MS vaccine gave higher immune response than single vaccine against mycoplasmosis infection in chickens and turkey poults, as it gave highest protection after vaccination. The use of combined vaccine also provided protection against more than one disease, decreased vaccination expenses and the number of vaccinations for every farm, saving time and decreasing the stress for birds.

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Authors' Contribution

All authors contributed equally.

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