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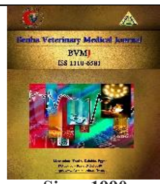

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Development and evaluation of an inactivated oil emulsion monovalent H5N8 Avian Influenza Vaccine for poultry

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ABSTRACT

Avian influenza (AI) is a highly contagious viral disease affecting poultry worldwide. It significantly impacts the poultry industry, causing economic losses. Developing effective vaccines is crucial to enhancing immune response and controlling infections. H5N8 is a subtype of highly pathogenic avian influenza virus (HPAIV), causing severe outbreaks in 2016–2017 across Egypt, Korea, Europe, and other regions. Mallards experienced high mortality at medium and high viral doses, while chickens required higher doses for fatal outcomes. Vaccination is the most effective strategy to control the spread of HPAI H5N8, helping to reduce infection rates and prevent outbreaks. This study focused on developing a monovalent inactivated oil-emulsion H5N8 vaccine using the Egyptian HPAI H5N8 strain (A/Chicken/Egypt/F71-586C/2022). Montanide ISA-71 VG served as the oil adjuvant. The final vaccine underwent sterility, safety, and potency testing. The potency of the prepared vaccine was evaluated by measuring the antibody response of vaccinated chickens using the HI test in addition to resistance to the challenge virus. It proved to be safe, sterile, and effective, with antibodies detected from the first week post-vaccination. Levels peaked at week three, reaching 10 $\log_2$, before declining to 8 $\log_2$ by the experiment's end. Vaccinated birds exhibited 90% protection against H5N8, showing mild clinical signs but no mortalities. Finally, the successful development of a safe and immunogenic inactivated H5N8 avian influenza vaccine formulated with Montanide ISA 71 VG significantly reduced viral shedding, which was completely eliminated within a few days post-challenge, making it an effective tool for controlling HPAI H5N8 outbreaks and reducing economic losses in the poultry industry.

1. INTRODUCTION

Type A avian influenza viruses (AIVs) are highly contagious pathogens classified under the Orthomyxoviridae family, widely infecting both avian and mammalian species (Swayne and Halvorson, 2003). The viral genome is single-stranded negative-sense RNA of 8 segments (Palese, 1980). They are enveloped viruses with surface glycoprotein projections that are hemagglutinin (HA) and neuraminidase (NA), which are subtypes of the influenza A virus and play crucial roles in the virus's ability to infect host cells and spread (Büchen-Osmond, 2003). Neutralizing antibodies against HA prevent viral attachment to host cells, with 18 known subtypes, while antibodies against NA, spanning 11 subtypes, provide a minor contribution to immunity (Swayne et al., 2000; Swayne and Halveron, 2008; Xiong et al., 2013; McAuley et al., 2019).

Highly pathogenic avian influenza virus (HPAIV) belongs to influenza type A viruses, especially subtypes H5 and H7 (Spackman, 2008), and causes outbreaks in Africa, Europe, and Asia, causing high mortalities and poultry destruction (OIE, 2017). The H5 subtype of HPAIV spread to wild birds, triggering the largest recorded epidemic in Europe in 2016–2017. It affected both poultry and wild species. During its transmission, the virus frequently reasserted itself with low-pathogenic avian influenza strains. H5N8 HPAIV is highly pathogenic, infecting various birds, including poultry, and causing severe economic losses. First detected in China in 2010, it belongs to clade 2.3.4.4. (Su et al., 2015).

First identified in wild birds in Asia in 2010, HPAIV (H5N8) strains of clade 2.3.4.4 subsequently infected domestic birds in China, South Korea, and Japan (Kang et al., 2014). A novel reassortant H5N8 virus from clade 2.3.4.4 spread throughout Europe, Asia, and the Middle East after its discovery in Russia. (Lee et al., 2017). HPAIV H5N8 was likely identified in Egypt through systematic avian influenza surveillance in wild migratory birds. As a key transit hub between Europe, Asia, and Africa, Egypt sees numerous migratory species passing through during their annual journeys (Denny, 1991; Webster et al., 1992).

HPAIV H5N8 was first detected in Egypt at Damietta Governorate, the same site where HPAIV H5N1 emerged in 2006 during its global spread (Saad et al., 2007). The virus later infected domestic poultry across several governorates (OIE, 2017). It was found in healthy, sick, and dead birds, but no human infections have been reported (Yehia et al., 2018).

Chickens infected with H5N8 HPAIV exhibited diverse clinical signs, including head shaking and ataxia, with mortality rates ranging from 5 to 70%. Post-mortem findings revealed hemorrhagic pneumonia and enteritis (Tarek et al., 2021; Amer et al., 2024) .

Vaccination decreases clinical signs and viral spread, preventing infection in other birds (Lee and Suarez, 2005). An effective vaccine raises the threshold for infection,

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serving as the first step in controlling disease transmission (Capua et al., 2003).

All avian influenza (AI) vaccines protect birds through mucosal and systemic humoral immunity against HA proteins (Swayne and Kapczynski, 2008). The effectiveness of AI vaccines depends on the age, the immune competency of the vaccine, and the antigenic relatedness between the vaccinal strain and the circulating virus strains (Fiore et al., 2009).

The inactivated vaccine is the most widely used for avian influenza. It is produced by propagating the virus in embryonated chicken eggs (ECE) and combining it with an adjuvant to enhance the immune response. This formulation ensures a high level of neutralizing antibodies (Garcia et al., 1998).

The inactivated vaccine requires a high antigen dose combined with an adjuvant to trigger protective immunity. While it induces a strong humoral response, its mucosal and cellular immunity remains relatively low (Wareing and Tannock, 2001).

Oil emulsion adjuvants are widely used in experimental inactivated AI vaccines for poultry. These emulsions enhance antibody production and prolong antigen release, leading to a stronger immune response (Liu et al., 2011; Dey et al., 2023).

The objective of this study is to develop and characterize a monovalent inactivated H5N8 avian influenza vaccine for poultry, employing a water-in-oil (W/O) emulsion system with Montanide ISA 70VG to enhance immunogenicity and protective efficacy.

2. MATERIAL AND METHODS

Ethical statement

The experiment was conducted following the guidelines of the animal welfare committee and the authorized Research Ethics Board of the Faculty of Veterinary Medicine at Benha University, registered at the national level under the code BUFVTM 06-02-23.

2.1. Avian influenza virus subtype H5N8

Egyptian HPAIV subtype H5N8 virus (A/Chicken/ Egypt/F71-S8C/2002) HPAIV-H5N8 2.3.4.4 that was kindly provided by Animal Health Research Institute (AHRI), Dokki, and Giza, Egypt. It was propagated on SPF-ECE and its titer was 107 EID50/ml and 8 log2 HAU/25 microliters. It was used as a reference seed stock and challenge virus. The AIV subtype H5N8 RG virus used in the HI test was obtained from the Reference Lab for Veterinary Quality Control on Poultry Production (RLQp).

Specific pathogen-free chicken eggs and birds served as the adaptation's host. H5N8V is the only virus utilized as a challenge. They were infected with 0.1 ml per bird intranasally and used as a challenge virus with a titer of 6 log10 EID50. (Spackman and Killian, 2014).

2.2. Fertile eggs

The Nile specific pathogen free eggs project, Kom Oshiem, EL-Fayoum, Egypt, is the source of the specific pathogen free embryonated chicken eggs (SPF-ECEs). The eggs were used for titration, viral propagation, and the procedure of ensuring that the virus was inactivated.

2.3. Chickens

One hundred chickens, each 21 days old and hatched from SPF-ECEs sourced from the Nile-SPF egg farm in Kom Oshiem, EL-Fayoum, Egypt, were used for the experiment. The chickens were floor-reared, fed on commercial poultry ration, and kept under strict hygienic measures in isolated brooder units throughout the experiment. The chickens were used for evaluation of the prepared vaccine.

2.4. Oil adjuvant

Montanide ISA 70VG was bought from SEPPIC S.A., 50 boulevard national, 92250, La Garenne-Colomes, France. Batch number 201118013951, Product code 36514P. It is a mineral oil-based adjuvant created to produce water-in-oil (W/O) emulsion. It has a special light mineral oil and a very pure emulsifier made from mannitol and cleaned vegetable oleic acid. It is free of animal origin.

2.5. Vaccine preparation

2.5.1. Virus propagation

The vaccinal seed virus strain AIV subtype H5N8 RG virus was diluted in sterile physiological normal saline to reach a titer of 103 EID50/dose to be inoculated into the allantoic cavity of 10-day-old SPF-ECEs with incubation at 37 °C for 72 hrs (Garcia et al., 1998). The inoculated eggs were subjected to daily candling, with the dead embryos being excluded in the first 24 hours of incubation as nonspecific deaths. After 72 hrs, the infected eggs were chilled at 4 °C and allantoic fluid was collected and stored at 4 °C after the addition of antibiotic solution. The collected fluid was left for one hour at room temperature for antibiotic action and then tested for sterility.

2.5.2. Titration of the propagated AIV

Serial tenfold dilutions of the virus were prepared. Each dilution from 3 log 10 to 10 log 10 was inoculated in 5 SPF-ECEs of 10 days old and incubated at 37 °C with daily candling for 6 days. Five SPF-ECEs were used as controls (none inoculated). Dead embryos during the first 24 hours were considered non-specific deaths and excluded. The allantoic fluid of the infected eggs was subjected to slide HA to detect the positive. Estimation of the virus titer as EID50 was calculated according to Reed and Munch (1938).

2.5.3. Virus inactivation

Virus inactivation was done by applying formalin (its concentration is 37-40%, and it was purchased from Sigma-Aldrich Laborchemikalien GmbH, Germany). Formalin was used in a final concentration of 0.1% of the total volume of the harvested viral allantoic fluid. Formalin was added to the viral fluid drop by drop during stirring, and after the addition of the formalin, the fluid was left with continuous stirring for 18 hours on the magnetic stirrer at 25 °C and then 2% of the total fluid volume of sodium bisulfite was added to stop the formalin reaction (OIE, 2004). The inactivated viral fluid was stored at 4 °C.

2.5.4. Completion of inactivation

Samples from the inactivated viruses before adding the adjuvant were tested for three passages in 10-day-old SPF-ECEs (0.2 ml/egg) via the allantoic cavity.

2.5.5. Preparation of the AIV oil emulsion vaccine

An aqueous phase containing the virus was prepared as a water-in-oil emulsion vaccine by using Montanide ISA 70 VG at a ratio of 30/70 (wt/wt). The preparation according to the SEPPIC protocol was carried out to prepare 100 mL of vaccine. The Montanide ISA 70 VG was mixed at 1500 rpm for 5-10 min, then the speed was increased to 3000 rpm for 20 min, then the aqueous medium was dispensed and mixed for 40 min under complete aseptic conditions, and the final product was stored at 4°C.

2.6. Quality control of the prepared vaccine

2.6.1. Sterility test

A sample of 0.5 ml of the final product vaccine was cultivated on nutrient agar (Oxoid), thioglycolate broth (Oxoid), and Sabouraud glucose agar (Oxoid) for the detection of bacteria, mycoplasma, and fungi, respectively, according to *Cruickshank et al. (1975).*

2.6.2. Safety test

The safety test involved injecting 1 ml (double dose) of prepared vaccines S/C at the neck of 21-day-old SPF chickens, followed by daily observation for 2 weeks to monitor both local and systemic reactions (OIE, 2008).

2.7. Experimental design

One hundred SPF chickens of 21 days old were divided into 2 groups with 50 chickens for each group. One group was vaccinated with the prepared vaccine in a dose of 0.5 ml subcutaneously at the nape of the neck, and the other group was a control, not vaccinated. Serum samples were collected from each group at regular time intervals for serological monitoring of antibodies. At 30 days post vaccination, ten chickens from the vaccinated group and ten chickens from the control group were isolated in isolators and were challenged against the local Egyptian AIV H5N8 strain by the intranasal route in a dose of 100 µl of allantoic fluid containing 106 EID50/bird. Oro-pharyngeal swabs were taken at days 3, 5, 7, and 10 post challenge and placed in 1 ml sterile physiological saline with an antibiotic-antifungal mixture to detect the virus shedding using real-time reverse transcription PCR (rRT-PCR).

2.8. Serum samples

Five random blood samples were obtained from each group weekly from the first week to the fourth WPV and then every two weeks until the 14th WPV. Blood samples were collected from the jugular vein and coagulated at 37ºC for an hour. They were then refrigerated at 4ºC overnight. Sera were separated by centrifugation at 1500 rpm for 10 minutes and stored in a dry and sterile tube at -20°C until serological analysis *(OIE, 2008).*

2.9. Serological monitoring of antibodies

The Hemagglutination Inhibition (HI) test was done on serum samples using AIV H5N8 subtype antigens for evaluation of the humoral immune response against the inactivated AIV H5N8 vaccine *(OIE, 2006).* First, determine the number of HA units of the reference virus using the Hemagglutination (HA) test. Chicken RBCs in a 1% (v/v) suspension were used for both HA and HI tests. Inactivation of sera was applied at 56ºC for 30 minutes before testing using the HI test. The antibody titer of the serum sample is calculated as the highest dilution of serum causing complete inhibition of 4 HA units of the virus *(OIE, 2006).*

2.10. Challenge of vaccinated chickens and detection of shed virus

Ten chickens from both the vaccine and control chicken groups were transferred separately to BSL3 biological isolators and were challenged against the local Egyptian AIV H5N8 strain (100 µl of 6 log10EID50 of challenge virus /chicken), intranasally *(OIE, 2008).* Chicken groups subjected to challenge were subjected to daily observation for 10 days post-challenge to record the clinical signs and mortalities with collection of tracheal and cloacal swabs at the 3rd, 5th, 7th, and 10th days post-challenge. For the detection of virus shedding using rRT-PCR.

2.11. Real-time reverse transcription PCR (rRT-PCR).

Viral RNA from tracheal and cloacal swabs of challenged chickens was extracted using the QIAamp Viral RNA Mini Kit, cat. No. 52904 (Qiagen, Hilden, Germany, GmbH), following the manufacturer's guidelines. Primers and probes supplied from Metabion (Planegg, Germany) targeting the AIV H5 subtype were used. The Primer/Probe Sequence 3-5 H5LH1-ACATATGACTACCCACARTATTCAG, H5RH1-AGACCAGCTAYCATGATTGC, and H5PRO-TCWACAGTGGCGAGTTCCCTAGCA [TAMRA][FAM] (Londt et al., 2008). Real-time RT-PCR amplifications were performed in a final volume of 25 µL containing 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 0.5 µl of forward primer (50 pmol), 0.5 µl of reverse primer (50 pmol), 0.125 µl of probe (30 pmol), 0.25 µl of QuantiTect RT Mix, 8.125 µl of RNase-free water, and 3 µl of template RNA. Reverse transcription was conducted at 50°C for 30 min, followed by primary denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 54°C for 30 s, and extension at 72°C for 10 s. The reaction was done with StepOne Plus, Applied Biosystems, Thermofisher real-time PCR equipment.

3. RESULTS

3.1. Virus propagation and titration

The avian influenza virus H5N8 was cultured in 10-day-old SPF embryonated chicken eggs. The virus titer in the harvested allantoic fluid reached 10 log7 EID50/ml, as measured by the infectivity titration test, and 8 log2 HAU/25 microliters, as determined by the hemagglutination test.

3.2. Inactivation and completion of inactivation of the seed H5N8 AIV

The seed virus in the harvested allantoic fluid from SPF-ECE following virus propagation was inactivated using 0.1% formalin for 20 hours. Testing samples taken from this inactivated seed virus confirmed the absence of any live virus, as no hemagglutination was observed after three successive blind passages in SPF-ECE.

3.3. Sterility and safety tests of the prepared vaccine

The sterility of the prepared vaccine was validated by culturing samples on specialized media designed to detect aerobic, anaerobic, and fungal growth. The analysis confirmed the absence of bacterial or fungal contamination, ensuring complete sterility. Additionally, safety testing demonstrated the vaccine's reliability, as no local or systemic reactions were observed following subcutaneous inoculation of 1 ml (double dose) into the necks of 21-day-old SPF chickens, with daily monitoring over two weeks.

3.4. Evaluation of the humoral immune response of vaccinated chicks using the HI test

The humoral immune response in chicks vaccinated with an inactivated monovalent influenza H5N8 vaccine containing Montanide ISA 70VG adjuvant was assessed using the HI test. The mean log_2 HI antibody titer in the vaccinated chicks group reached 5 log_2 during the first- and second-weeks post-vaccination. By the third week, titers increased to 10 log2, indicating a strong immune response. The antibody levels remained at 7 log_2 till the end of the experiment in the vaccinated group. A comparison of HI antibody titers induced by the vaccine in vaccinated chicks versus the control group is presented in Table (1).

Table 1 Mean log2 HI antibody titers of chicks vaccinated with the inactivated monovalent influenza H5N8 vaccine with Montanide ISA 70VG adjuvant.

Group	Mean log2 HI antibody titer/weeks post vaccination								
	W 1	W 2	W 3	W 4	W 6	W 7	W 9	W 11	W 14
Vaccinated	5	5	10	8	8	8	7	7	7
Control	0	0	0	0	0	0	0	0	0

3.5. Evaluation of protection percentage of vaccinated chicks

The protective efficacy of the inactivated monovalent influenza H5N8 vaccine was evaluated through a challenge test using a virulent reference strain of H5N8, 30 days post-vaccination. The vaccinated chicks exhibited an 80% protection rate, whereas the non-vaccinated control group showed 0% protection, highlighting the vaccine's effectiveness. Results are detailed in Table 2.

Table 2 Protection percentage of chicks vaccinated with inactivated monovalent influenza H5N8 vaccine.

Group	No. of birds	Total morbidities	Total mortalities	Protection %
Vaccinated	10	2	0	80%
Control	10	10	10	0

3.5. Evaluation of AIV H5N8 shedding using RRT-PCR in vaccinated chicks following challenge

Real-time RT-PCR was used to analyze the shedding of AIV H5N8 in challenged chicks immunized with an inactivated monovalent H5N8 influenza vaccine prepared with Montanide™ ISA 71-RVG oil adjuvant. Viral shedding was identified on the third day after challenge, but in vaccinated chicks, it was negative on the fifth, seventh, and tenth days. In contrast, the control non-vaccinated group excreted 100% of the virus during the observation period (Table 3).

Table 3 Evaluation of virus shedding after challenge with AIV H5N8 of chicks vaccinated with monovalent influenza subtype H5N8 vaccine real-time RT-PCR.

Days Post challenge	Group	Result	C.T	Shedding Amount
3	Vaccinated	+ ve	26.33	$4.639X\ 10^3$
	Control	+ ve	15.25	$1.042X\ 10^7$
5	Vaccinated	-ve	No	No
	Control	Dead	No	No
7	Vaccinated	-ve	No	No
	Control	Dead	No	No
10	Vaccinated	-ve	No	No
	Control	Dead	No	No

4. DISCUSSION

Outbreaks of highly pathogenic avian influenza virus (HPAIV) result in significant death rates among domestic chickens, causing severe economic losses in the poultry sector. (Swayne and Suarez 2000). In 2010, China experienced outbreaks of a newly emerged virus affecting both wild and domestic poultry, identified as the HPAIV H5N8 subtype. That same year, a reassortant H5N8 virus was isolated from a mallard duck in China (Zhao et al., 2013). Since 2014, the H5N8 virus has triggered outbreaks in Korea, Japan, and multiple European countries, affecting both wild and domestic poultry populations (Verhagen et al., 2015). The H5N8 virus is among the most significant HPAIV. Migratory birds have played a crucial role in its transmission, facilitating its spread across various regions. Additionally, aquatic birds serve as the primary natural reservoir, sustaining the virus in wild populations (Wester et al., 1992).

The recently emerged highly pathogenic avian influenza (HPAI) H5N8 subtype was identified in Egypt's Damietta Governorate through systematic surveillance of avian influenza in wild birds (Saad et al., 2007). As Egypt serves as a key stopover for various migratory bird species, its geographical position plays a significant role in the virus's monitoring and potential spread (Denny, 1991).

This study aimed to develop an inactivated monovalent H5N8 oil-emulsion vaccine, recognizing vaccination as the most effective strategy for reducing viral shedding and limiting the spread of infection (Capua et al., 2003). To develop an effective vaccine, selecting the most suitable adjuvant is crucial for achieving a strong and long-lasting immune response. In this study, Montanide ISA 71 VG was chosen as the optimal oil-based adjuvant for formulating the H5N8 vaccine, ensuring enhanced immunogenicity and prolonged protection (Seppic, 2002).

This study was conducted to develop a water-in-oil (W/O) emulsion formulation of the H5N8 vaccine and evaluate its efficacy based on immune response, as well as its ability to prevent clinical signs and reduce viral shedding. Initially, the H5N8 virus was propagated in 10-day-old embryonated chicken eggs (ECE) via the allantoic cavity, resulting in an infectivity titer of 10^7 EID50/ml and a hemagglutination activity of 8 log2 HAU/25 microliters.

The harvested fluid was inactivated using 0.1% formalin. To confirm complete inactivation, 0.2 ml of the treated fluid was inoculated into five embryonated chicken eggs (ECE) for three successive blind passages. Observations revealed no presence of live viruses, verifying the effectiveness of the inactivation process (Abd-Elwanis et al., 2008).

The vaccine was formulated using Montanide ISA 71 VG as an oil adjuvant, following manufacturer guidelines for vaccine preparation. After production, it underwent sterility testing to detect potential bacterial or fungal contamination, confirming that the vaccine was completely sterile (OIE, 2004). The prepared vaccine underwent safety testing by administering a double dose to chickens, followed by 14 days of observation for any local or systemic reactions. The results indicated no signs of local lesions or systemic effects, confirming the vaccine's safety.

The trial included 100 specified pathogen-free (SPF) hens, each 21 days old. They were separated into two groups of fifty birds each. The first group received the prepared H5N8 vaccine, while the second group remained unvaccinated, serving as the control group. Serum samples were collected at regular intervals from both groups to conduct the hemagglutination inhibition (HI) test, which allowed for the detection and measurement of antibody titers against the virus (OIE, 2006).

The HI test results showed the presence of H5N8-specific antibodies in the serum of vaccinated chickens from the first week post-vaccination, with a titer of 5 log2. The antibody levels peaked at 10 log2 by the third week, followed by a gradual decline to 7 log2, which persisted until the end of the experiment, and that agreed with Treanor et al. (2005), who said that infection with AIV leads to production of antibodies against HA and NA glycoproteins during the first 2 weeks after infection and the titer attains a peak for 4-7 weeks. A high titer of antibodies against the HA protein is related to good protection against the disease (Clement et al., 1986). It was revealed that Montanide ISA 71 VVG oil adjuvant induces an early immune response from the first week post vaccination, and this agrees with what was found by Kydyrbayev et al., 2010,

We estimated the protection percentage of the prepared vaccine by observing the challenged birds for 2 weeks, and we noted that the protection percentage was 80%, as 2 birds of 10, which were vaccinated, showed slight respiratory signs with depression and decreased feed intake without death cases. And by histopathological examination of internal organs of these tired birds, we found tracheitis associated with mild apoptosis of tracheal epithelium and focal hemorrhage. Also, the liver of these birds showed a decrease in the congestion of the liver sinusoids and mild degeneration of hepatocytes, but the kidney suffered from mild renal tubular degenerative changes and minute foci of mononuclear inflammatory cells with lymphoid hyperplasia of the white pulp of the spleen and a mild degree of lymphoid depletion of the follicle in cecal tonsils. These results are consistent with the results of Pasick (2004), which revealed that a single dose of vaccine has been shown to reduce mortalities in vaccinated birds following challenge without viremia and low viral shedding, but not eliminated.

Detection of viral shedding is important to evaluate the vaccine efficacy and control infection transmission (El-Masry et al., 2014), so we applied a challenge test on vaccinated chickens. Chickens were inoculated with 100 µl of allantoic fluid containing 105-106 EID50/bird by the intranasal route at day 30 post-vaccination, and oropharyngeal swabs were collected at days 3, 5, 7, and 10 post-challenge for detection of the amount of viral shedding from the vaccinated and control groups (OIE, 2008). The quantity of viral shedding in the vaccinated group challenged with the H5N8 virus was minimal on day 3 and thereafter eliminated. While birds in the control non-vaccinated group and those challenged with the same virus strain showed a high amount of viral shedding on day 3 post-challenge and then died, this result was consistent with OIE, 2012, which noted that vaccination with a monovalent vaccine reduces virus shedding, as well as Lee and Suarez (2005), who stated that vaccines with a specific level of antigen decrease viral excretion.

5. CONCLUSIONS

The present study demonstrated the successful development of a safe and immunogenic inactivated H5N8 avian influenza vaccine formulated with Montanide ISA 71 VG. The vaccine induced an early antibody response detectable from the first week post-vaccination and maintained protective titers throughout the experiment. Upon challenge, vaccinated chickens showed 80% protection with only mild clinical signs in a few birds and markedly reduced pathological changes compared to controls. In addition, the vaccine significantly minimized viral shedding, which was completely eliminated within a few days post-challenge, while unvaccinated birds continued to shed virus and succumbed to infection. These findings highlight the potential of the prepared vaccine as an effective tool for controlling HPAI H5N8 outbreaks and reducing economic losses in the poultry industry. Its use in vaccination programs could contribute to limiting viral spread, particularly in Egypt where migratory birds play a major role in introducing and maintaining avian influenza viruses.

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