Special Issue:

Veterinary Medicine between Sustainable Development and Public Health to Confront Global Changes

Assessment of Inactivated H5N8 Avian Influenza Vaccine Using Multiple Mucosal Adjuvants in Different Ways

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Abstract | Vaccination against Highly Pathogenic Avian Influenza" (HPAI) is the effective control practices in endemic countries to minimize the expected economic losses and human threats. Currently AI inactivated vaccine depend on using classical Oil excipients as adjuvants. We have formulated a novel monovalent experimental vaccine against the recent circulated HPAI H5N8 clade 2.3.4.4b viruses using following adjuvants (MONTANIDE™ GEL 01 PR, MONTANIDE™ IMS 1313, and MONTANIDE™ ISA70 to evaluate their impact on the vaccine efficacy, potency, with special focus to humoral and cell mediated immune response. Seven groups of specific pathogen-free (SPF) chickens were treated as G1 and G2 vaccinated with (H5N8+Gel 01), G3 and G4 with (H5N8+IMS1313) by intranasal (IN) and intramuscular (IM) for each treatment respectively. Each group (G1-G4) was vaccinated twice at day 7 and 21 of age. Meanwhile, G5 treated with (H5N8+ISA70) received the primary vaccination dose intramuscularly only on day 7 of age. Group G6 served as a positive control (untreated and challenged), and G7 served as a negative control (untreated and unchallenged). Chickens were challenged at day 35 of age with 10^6 EID50 of widely circulating H5N8 strain in Egypt to evaluate vaccine potency, efficacy, and safety. Results indicated that polymer GEL01/IM G2 and ISA70/IM G5 successfully conferred a high humoral immune response, reflecting 85% and 90% protection post- challenge respectively. ISA70/IM G5 also significantly enhanced mucosal immune responses (IgA level) post-challenge. In contrast, groups receiving intranasal vaccine (G1 and G3) showed unacceptable humeral immune response with protection rates of 45% and 15%, respectively. IMS1313 (G4) provided 0% protection accompanied with substantial increase in IL-6, IL-2, IL-4, and IFN-γ levels post-challenge, indicative of acute infection. We conclude that the vaccine adjuvant quality plays the corner stoon in initiation, magnitude and longevity of the specific immune response against target disease. Both GEL01/IM and ISA70/IM have demonstrated robust potency and efficacy. However, the superiority of ISA70 but the mucosal GEL01 adjuvant still showing promising results and space of improvement is required for the inactivated vaccine manufacturing. To achieve the highest level of protection, further studies on dual vaccination strategies involving both intranasal and intramuscular administration of different adjuvanted polymer is needed for optimizing the vaccine's efficacy against HPAI.

Keywords: H5N1, H5N8, HPAI, Vaccines, Mucosal Vaccines, Influenza Immunity

Received | May 10, 2024; **Accepted** | July 17, 2024; **Published** | August 15, 2024
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Citation | Ali I, Abdien HMF, Elfeil WK, ElDemerdash MMZ, El-Abideen MAZ, Kilany WH (2024). Assessment of inactivated H5N8 avian influenza vaccine using multiple mucosal adjuvants in different Ways. Adv. Anim. Vet. Sci. 12(s1): 37-48.
DOI | https://dx.doi.org/10.17582/journal.aavs/2024/12.s1.37.48
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

Avian influenza (AI) poses a major economic threat to the poultry industry worldwide, leading to substantial financial losses. Belonging to the *Orthomyxoviridae* family, genus *Orthomyxovirus*, AI viruses are characterized by a negative-sense, single-stranded, segmented RNA genome. Antigenically classified based on their hemagglutinin (HA) and neuraminidase (NA) spike proteins into 18 HA and 11 NA variants (Davis, 2014; Fiala *et al*., 2018; Naguib *et al*., 2019). Highly pathogenic avian influenza viruses (HPAIVs), particularly subtypes H5 and H7, pose significant threats to poultry due to their high mortality rates and zoonotic potential (Alexander, 2007; Bialy and Shelton, 2020). Mortality rates in infected poultry flocks can reach 100%, affecting economic and food security (Perkins and Swain, 2003). While human infections with AI viruses are rare in the Middle East and Africa, they can be fatal and usually occur through direct contact with infected poultry (Chen *et al*., 2005; Peiris *et al*., 2007). The emergence of H5N8 clade 2.3.4.4 in domestic poultry in China around 2009-2010 marked the beginning of its global spread, leading to subsequent outbreaks in South Korea, North America, Asia, Europe, Africa, and the Middle East by 2016 (Lee *et al*., 2015). Vaccination is critical to control avian influenza infections in poultry however, challenges persist due to viral mutations and mismatches between vaccine strains and circulating field viruses (Kandeil *et al*., 2018). Current inactivated vaccines primarily induce humoral immune responses but may insufficiently induce mucosal immunity, which is critical for preventing respiratory infections (Kang *et al*., 2004). Mucosal vaccination strategies, such as intranasal administration, have emerged as promising alternatives. These strategies enhance both local and systemic immune responses, making them effective for mass vaccination campaigns during disease outbreaks (Atmar *et al*., 2007; de Geus *et al*., 2011; De Wit *et al*., 2010; El Naggar *et al*., 2017; Worrall *et al*., 2009). Under normal conditions, inhaled antigen induces a state of tolerance rather than strong immune responses when it meets the respiratory tract mucosa (Akbari *et al*., 2001; El Naggar *et al*., 2017; Kapczynski *et al*., 2013) resulting in a tolerogenic mucosal environment. The intranasal application of the whole inactivated virus alone has poor immunogenicity (Akbari *et al*., 2001; El Naggar *et al*., 2017; Hagenaars *et al*., 2008; Tseng *et al*., 2009). Sodium polyacrylate gel particles in water form, known as the polymer-based adjuvant MONTANIDE™ Gel 01 ST (Gel 01) and MONTANIDE™ IMS 1313 N, are aqueous nanoparticles containing an immunostimulants. These adjuvants enhance the immunogenicity of fully inactivated viruses by boosting the local mucosal immune response at the site of entry, thereby inhibiting virus multiplication (Ismail *et al*., 2018). They have demonstrated effectiveness for large-scale vaccination and are suitable for intensive poultry operations, whether applied via shower, spray, or water source (Branton *et al*., 2005; Deville *et al*., 2012; El Naggar *et al*., 2017; Riffault *et al*., 2010). The goal of this study is to approve concept of mucosal inactivated AI vaccine against the HPAI H5N8 clade 2.3.4.4b using two mucosal adjuvants, MONTANIDE™ GEL 01 and MONTANIDE™ IMS 1313 either intranasally or intramuscularly compared to the oil-based MONTANIDE™ ISA70 vaccine. The study will assess the safety, efficacy, and immunogenicity of these vaccines by measuring mucosal, cellular, and humeral immune responses and their ability to protect against the HPAI H5N8 challenge.

MATRIALS AND METHOD

Viruses

Viral vaccine: Reassortant H5N8 (rgA/chicken/ME-2018/H5N8) (Accession n° MW193074) belonging to clade 2.3.4.4b was developed by MEVAC - Middle East for Vaccines, Egypt (Ibrahim *et al*., 2021). The vaccine strain was used for producing the antigen bulk for vaccine preparation through its propagation in 9-11 embryonated SPF chicken eggs (Nile SPF eggs, Kom Oshiem, Fayoum, Egypt) via allantoic sac (AS) route, incubated at 37°C and 65% RH for 72 hrs.

Challenge virus: HPAI strain [A/chicken/Qalubia/ME-VACF33/2017(H5N8)] (Accession n° MH349012) belonging to clade 2.3.4.4b was used for challenge experiment.

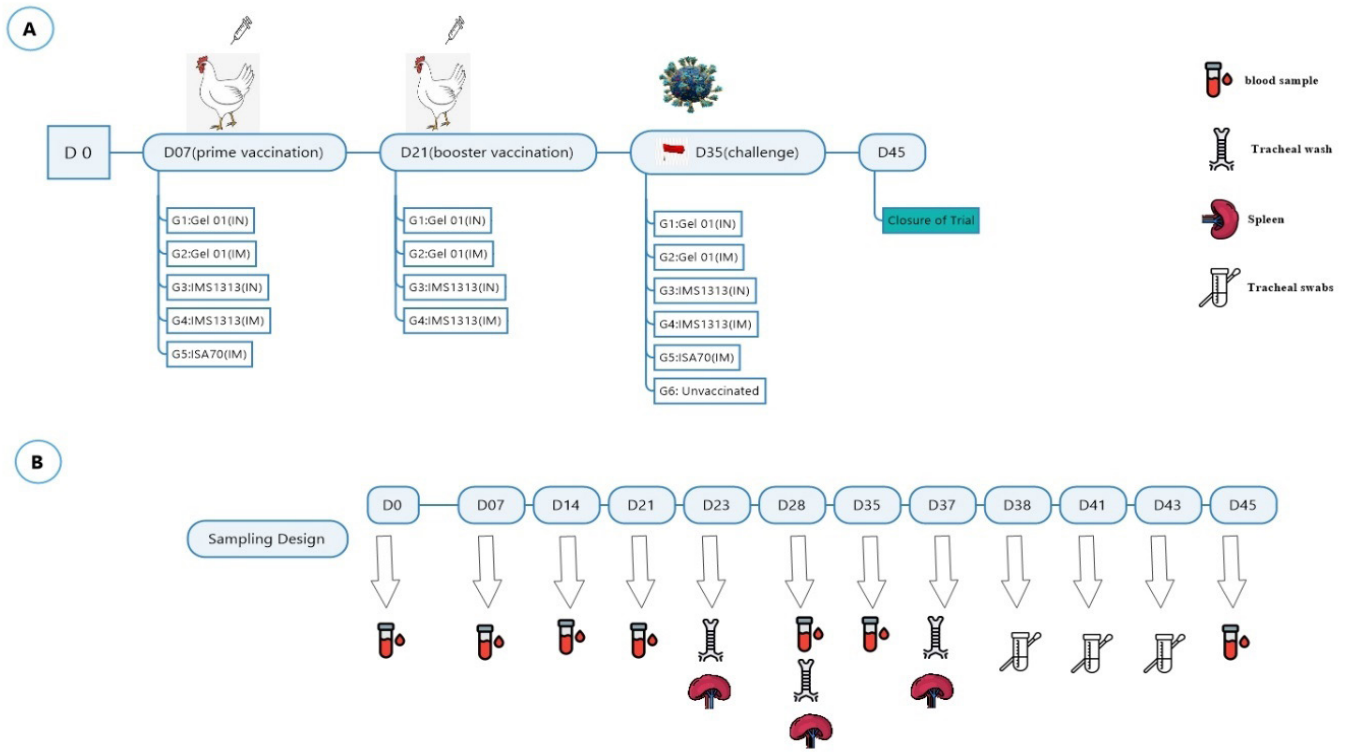

Figure 1: The experimental design for potency and efficacy testing (A) Schematic plane of the experimental design to evaluate the efficacy of various adjuvanted inactivated H5N8 AI vaccines (B) Sampling outline of the parameters evaluated during the experiment.

Preparation of vaccinal virus antigen

The vaccine seed virus was propagated by inoculating SPF Embryonated Chicken Eggs (ECE) via AS route for 10 days. The inoculated eggs were kept at 37°C and 65% RH for 72 hrs. After that, the infected allantoic fluid (AF) was collected and clarified using a low-speed centrifuge at 2000 rpm for 10 minutes at 4°C (WOAH, 2021). Subsequently, the harvested bulk virus was titrated to determine HA $\log^2$ titers and calculate the embryo infectious dose 50 (EID_{50}) (Karakus *et al*., 2018; Reed and Muench, 1938).

Virus Inactivation and neutralization

The bulk virus harvest was inactivation by adding 0.2% formaldehyde solution per Liter. The bulk virus inactivation was confirmed by adopting three successive passages using five SPF-ECE (10-day-old)/passage inoculated/ passage using the AS route. The inoculated SPF-ECE were then incubated at 37°C and 65% RH for 72 hrs. Slide hemagglutination (HA) testing was conducted to assess HA positivity. This involved adding an equivalent volume of allantoic fluid containing 10% washed chicken red blood cells (WOAH, 2021).

Vaccines preparation

The different vaccines were prepared following the World Organization for Animal Health (WOAH) manual of diagnostic tests and vaccines for terrestrial animals (Kilany *et al*., 2016; WOAH, 2021). The inactivated bulk virus antigen was mixed with different adjuvants according to the manufacturer's instructions: Montanid Gel 01, composed of a 20% gel phase and 80% water phase (v/v); IMS 1313, which consists of a 50% oil phase and 50% water phase (v/v); and Montanid ISA 70 Oil, comprising a 30% water phase and 70% oil phase (v/v) (SEPPIC, 2012). Emulsification of the vaccines was achieved using the Silverson L5M high-shear laboratory mixer (Silverson Machines, Inc., Buckinghamshire, United Kingdom). The temperature of the mixture was carefully maintained between 18°C and 22°C throughout the emulsion formulation process (Kilany *et al*., 2016).

Birds and experimental design

A total of 210 one-day-old SPF chicks (Nile SPF eggs, Kom Oshiem, Fayoum, Egypt) were randomly allotted into seven groups (n=30 chicks/group). They were individually housed in chicken isolators at the BSL-3 Facility for Laboratory Animals, MEVAC - Middle East for Vaccines, Egypt, and provided with ad libitum food and water. Bird groups, vaccination strategies, and sampling taken were summarized in Figure 1. The positive control group was exposed to virus challenge conditions while the negative control group received sterile PBS injections without treatment.

Challenge experiment

On day 35 of age, six groups of chickens [G1-G6] were intentionally exposed to 100µl ($6\log_{10}$ EID_{50}) of HPAI H5N8 virus subtype 2.3.4.4b/Chick through oculonasal instillation. Over the next ten days post-challenge (dpc), we closely monitored the chickens to evaluate the safety and efficacy of the vaccine. This comprehensive assessment included observing any clinical signs of HPAI, as well as evaluating mortality and survival rates, level of protection, and virus shedding. Tracheal swabs were collected at 3, 6, and 8 dpc in 1 mL PBS (pH 7.2-7.4) to determine the level of virus shedding by the EID50 method. Subsequently, the collected swabs were centrifuged to remove debris, and the resulting supernatants were then diluted using sterile PBS. The diluted samples were carefully titrated into embryonated SPF chicken eggs, incubated at 37°C with 55-60% RH, and examined for hemagglutinating activity after 3 days (Karakus *et al*., 2018).

Measured parameters

Measurement of humoral immune response

Serum samples (n=15/Group) were collected weekly from days (D0 to D43) to measure the humoral immune response by hemagglutination inhibition (HI) assay using the diagnostic avian influenza viruses H5N8 subtype clade 2.3.4.4b (Karakus *et al*., 2018). In the HI assay, four hemagglutination (HA) units of diagnostic antigen were combined with twofold serial dilutions of sera. A 1% suspension of red blood cells (RBCs) was used, and the HI titer was measured. The arithmetic means of HI titers were represented as reciprocal $\log^2$ values. A hemagglutination suppression at a dilution ≥ $2\log^2$ was considered as a specific positive antibody for AIV.

Measurement of cell-mediated and local immune response

The expression levels of interleukins (IL-2, IL-4, IL-6) and interferon (IFN-γ) mRNA in spleen tissues of the experimental chicks were measured at 2 and 7 days post-vaccination (dpv) and 2 days post-challenge (dpc) using a quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) kit: AgPath-ID™ One-Step RT-PCR Reagents, with the RNA extraction performed using the Easy Pure® Simple Viral DNA/RNA Kit. The primers and thermal conditions are detailed in Tables 1 and 2, (Kaiser et al., 2003; Peters et al., 2003; Rothwell et al., 2004). In addition, the IgA expression levels were measured in tracheal lavage using an Enzyme-Linked Immunosorbent Assay (ELISA) with the chicken IgA ELISA Core Kit, as per the manufacturer's instructions (Catalog No. K0231034). Spleen samples and tracheal lavage were collected on RNAlater® (ThermoFisher Scientific, UK). (Kaiser *et al*., 2003; Peters *et al*., 2003).

Statistics

The data was analyzed using SPSS version 21.0 (2016). A one-way ANOVA was performed to assess the statistical significance of group differences. After that, Duncan's new multiple-range test was used to evaluate the significance of variations between specific treatments and comparable controls.

Table 1: Cytokines Oligonucleotide primers and probes

Primer/Probe	Sequence
IL-2 probe1 (Kaiser *et al*., 2003)	[FAM] 5'-ACT GAG ACC CAG GAG TGC ACC CAG C-3' [TAMRA]
IL-2 F1	5'-TTG GAA AAT ATC AAG AAC AAG ATT CAT C-3'
IL-2 R1	5'-TCC CAG GTA ACA CTG CAG AGT TT-3'
IL-4 probe1 (Rothwell *et al*., 2004)	[FAM] 5'-AGC AGC ACC TCC CTC AAG GCA CC-3' [TAMRA]
IL-4 F1	5'-AAC ATG CGT CAG CTC CTG AAT-3'
IL-4 R1	5'-TCT GCT AGG AAC TTC TCC ATT GAA-3'
IL-6 probe1 (Kaiser *et al*., 2003)	[FAM] 5'-AGG AGA AAT GCC TGA CGA AGC TCT CCA-3' [TAMRA]
IL-6 F1	5'-GCT CGC CGG CTT CGA-3'
IL-6 R1	5'-GGT AGG TCT GAA AGG CGA ACA G-3'
28S rRNA probe1 (Peters *et al*., 2003)	[FAM] 5'-AGG ACC GCT ACG GAC CTC CAC CA-3' [TAMRA]
28S rRNA F1	5'-GGC GAA GCC AGA GGA AAC T-3'
28S rRNA R1	5'-GAC GAC CGA TTT GCA CGT C-3'
IFN-γ probe1 (Kaiser *et al*., 2003)	[FAM] 5'-TGG CCA AGC TCC CGA TGA ACG A-3' [TAMRA]
IFN-γ F1	5'-GTG AAG AAG GTG AAA GAT ATC ATG GA-3'
IFN-γ R1	5'-GCT TTG CGC TGG ATT CTC A-3'

Table 2: Thermocycling condition using in qRT-PCR.

Stage	Temperature	Time	Cycles
Reverse Transcription	50 °c	30 min	1
Primary denaturation	94 °c	10 min	1
Amplification Secondary denaturation	94 °c	15 sec	40
Annealing and extension	60 °c	1 min	

RESULTS AND DISCUSSION

Humoral immune response after vaccination

Postvaccination humoral antibody response against H5N8 2.3.4.4b were carefully monitored in chickens after vacci-

nation on weekly basis. Two weeks after the first dose and two weeks after the booster dose. In the group G5 (ISA70/IM) which did not receive a booster dose, the antibody levels started to increase after 3 weeks of vaccination, while the negative control group SPF (G7) remained negative for HI antibodies. Our results indicated that the highest HI antibody titers were observed in groups G2 (GEL01/IM) and G5 (ISA70/IM) where the differences in HI antibody titers were statistically significant compared to other groups ($p < 0.001$). In G2, the titers increased during the first- and second-weeks post- booster dose (log^2 6.5±2.6 and log^2 7.8±2.2, respectively). In G5, the titers increased after 2- and 3-weeks post-vaccination (log^2 6.25±1.9 and log^2 8.3±1.4, respectively) and reached the maximum after four weeks of vaccination (8.5±0.9). Conversely, the titers were less than log^2 2.5±1.4 in the other groups G1(GEL01/IN), G3 (IMS1313/IN), and G4 (IMS1313/IM) (Figure 2).

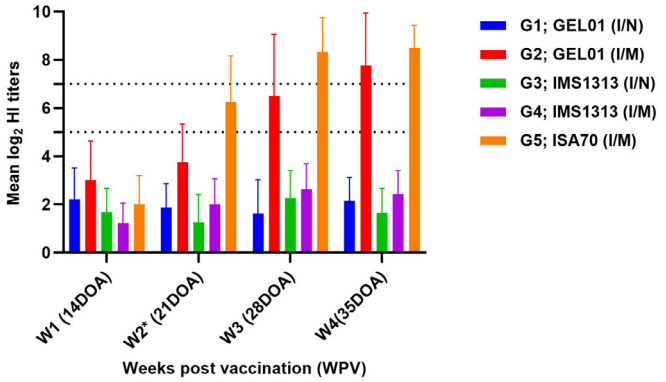

Figure 2: Mean serum hemagglutination inhibition titers of HPAI H5N8 virus (log2) of vaccinated chicken groups weekly.

Cell-mediated immune response post-vaccination

***mRNA* expression levels of cytokines:** The mRNA expression levels of IL-2, IL-4, IL-6, and IFN-γ in splenocytes did not demonstrate a statistically significant increase after vaccination in all vaccinated groups at 2- or 7-days post-vaccination (dpv) according to a two-way ANOVA test ($p > 0.05$) Nonetheless, IL-2 was upregulated 2.9, 2.7, and 1.4 times after 2dpv in the G5 (ISA70/IM), G2 (GEL01/IM), and G3 (IMS1313/IN) vaccinated groups respectively, and by 1-fold after 7dpv for both G2 and G5 compared to the negative control group (Figure 3).

The mRNA expression levels of IL-4 increased after 2 dpv by 36.5-fold with G4 (IMS1313/IM), 17.4-fold with G3 (IMS1313/IN), 10.7-fold with G5 (ISA70/IM), and 9.7-fold with G2 (GEL01/IM) compared to the negative control. After 7 dpv, the expression levels numerically elevated by 211.2-fold in G1 (GEL01 IN), 125.9-fold in G5 (ISA70 IM), and 123.9-fold in G2 (GEL01 IM), while it persisted with a 17.4-fold increase in G3 (IMS1313/IN) compared to the negative control group (Figure 3).

Interleukin-6 mRNA expression levels were upregulated by 4.9-fold after 2dpv with G4(IMS1313/IM) vaccinated group, while they were elevated by 78.8, and 77.4-fold with G5(ISA70/IM), and G2(GEL01/IM) vaccinated groups respectively compared to the negative control group (Figure 3).

The mRNA expression of interferon-gamma showed a slight 6.5-fold increase with G4 (IMS1313/IM) at 2dpv and elevated 38.7-fold with G4 (IMS1313/IM), 28-fold with G5 (ISA70/IM), and 26.6-fold with G2 (GEL01/IM) vaccinated groups at 7dpv, compared to the negative control (Figure 3).

Local immune response post-vaccination

***IgA* antibody levels:** Following vaccination, there was a significant 692-fold ($P < 0.05$) increase in the levels of secretory IgA antibodies specific for influenza AIV virus (sIgA) in the tracheal lavage of experimentally vaccinated chickens with G5 (ISA70/IM) at 7 days post-vaccination while they were non-significantly increased by 283-fold at 2 days post-vaccination. Groups G1, G4, and G2 showed non-significant increases of 137-, 132- and 15-fold respectively at 7 days post-vaccination, compared to negative controls (Figure 4).

Challenge experiment

Protection percentage and survival rate post-challenge: The negative control group (G7) remained free of clinical symptoms throughout the trial. In contrast, the SPF non-vaccinated challenged control chicks (G6) exhibited typical avian influenza clinical symptoms, including edema, cyanosis of the comb and wattles, and blood spots on leg shanks just 2 days after the challenge, with a mortality rate of up to 100% at 3 days post-challenge. Among the vaccinated challenged groups, the G2 (GEL01/IM) and G5 (ISA70/IM) groups displayed remarkably high protection levels of up to 85% and 90% respectively, followed by G1 (GEL01/IN) and G3 (IMS1313/IN) with partial and lower protection levels of 46% and 15%. However, the G4 (IMS1313/IM) group showed zero protection post-challenge (Figure 3).

Virus shedding post-challenge

Table 3, shows the percentage of HPAI H5N8 virus shedding in tracheal swabs for experimental groups after the challenge which was examined by EID50 on all intervening days. All vaccinated challenged chicken groups (G1 to G5) showed a decrease in virus shedding titres (0.5, 0.5, 0.2, 0.3, 0.3 log^{10}) respectively at 6dpc. No viral shedding was detected in groups G1(GEL01/IN),

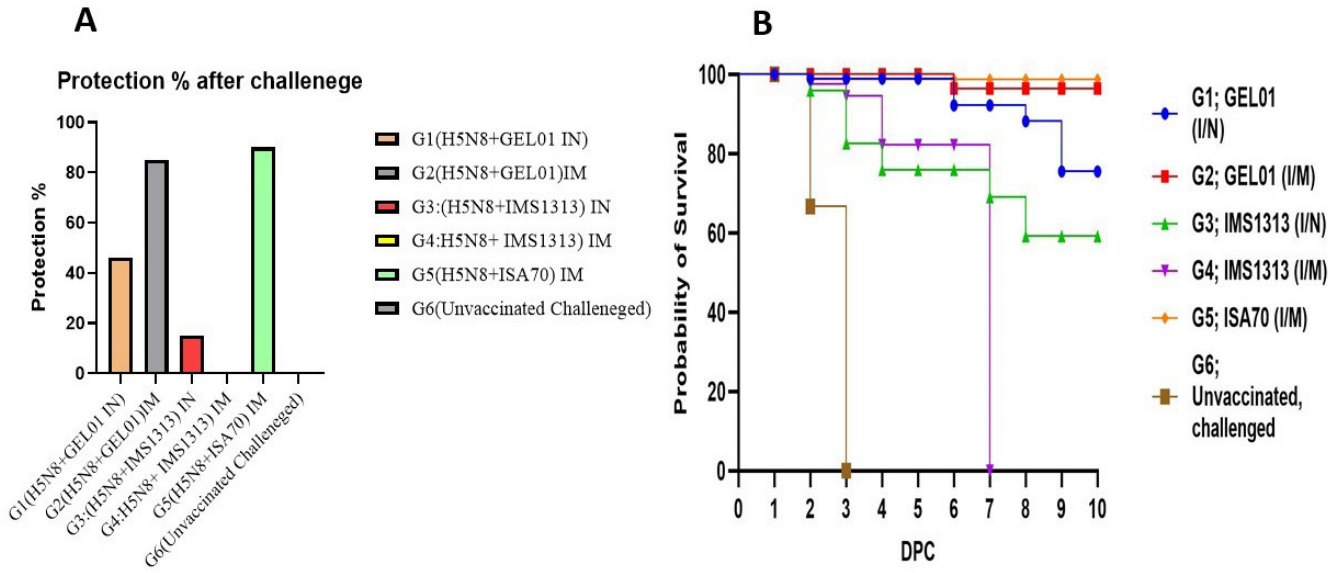

Figure 3: (A) Protection percentage (B) survival curve of chicken vaccinated groups after challenge with HPAI-H5N8.

G3(IMS1313/IN), and G5(ISA70/IM) while G2(GEL 01/IM) showed a 2.2 $\log^2$ decrease in shedding titres at 8dpc (Table 3, Figure 3).

Table 3: Results of shedding ratio and titters in vaccinated chickens at intervening days after challenge with HPAI-H5N8

Groups	Days pos- challenge (DPC)		
	3DPC	6DPC	8DPC
G1	4/10 (40%) 7.4 $\log_{10}$ EID_{50}/mL	3/9 (33%) 6.9 $\log_{10}$ EID_{50}/mL	0/6 (0%)
G2	2/10 (20%) 6.9 $\log_{10}$ EID_{50}/mL	4/10 (40%) 6.4 $\log_{10}$ EID_{50}/mL	2/9 (22%) 4.2 $\log_{10}$ EID_{50}/mL
G3	3/7 (42%) 7.4 $\log_{10}$ EID_{50}/mL	2/3 (66%) 7.2 $\log_{10}$ EID_{50}/mL	0/2 (0%)
G4	6/7 (85%) 8.2 $\log_{10}$ EID_{50}/mL	4/4 (100%) 7.9 $\log_{10}$ EID_{50}/mL	N/A (All dead)
G5	2/9 (22%) 8.2 $\log_{10}$ EID_{50}/mL	2/9 (22%) 7.9 $\log_{10}$ EID_{50}/mL	0/9 (0%)
G6	N/A (All dead)	N/A	N/A
G7	0/9 (0%)	0/9 (0%)	0/9 (0%)

Measured outcomes of cell-mediated and local immune response 2dpc

Cytokines expression levels (2dpc): IL-2 mRNA expression levels significantly increased by 76.3-fold (P****<0.0001) with G1(GEL01/IN), 40.8-fold (P*<0.05) with G4(IMS1313/IM), and 24.3-fold with G3(IMS1313/IN) compared to the negative control group (Figure 4).

IL-4 mRNA expression level was significantly higher by 424.2-folds (P*<0.05) with G4(IMS1313/IM) and slightly increased by 37.9, 35.9, 20.5 and 18.1-folds with G5(ISA70/IM), G2(GEL01/IM), G1(GEL01/IN) and G3(IMS1313/IN) vaccinated groups respectively compared to the negative control (Figure 4).

IL-6 mRNA expression level significantly increased by 31096-fold with the G4(IMS1313/IM) group (P**<0.01). Non-significant increases were observed by 16199, 887, 148, and 139.7-folds with G1(GEL01/IN), G3(IMS1313/IN), G2(GEL01/IM), and G5(ISA70/IM) vaccinated groups respectively compared to the negative control (Figure 4).

There was a significant regulated increase in IFN-γ mRNA expression level by 925 (P**<0.01) with G4(IMS1313/IM) and by 641-folds with G3(IMS1313/IN) (P*<0.05). Additionally, a non-significant, numeric increase by 293, 65, and 63-folds with G1(GEL01/IN), G5(ISA70/IM), and G2(GEL01/IM) vaccinated groups respectively were detected compared to the negative control group (Figure 4).

IgA* local antibody levels (2dpc):** The chickens vaccinated with ISA70/IM (G5) showed a highly significant increase of local sIgA antibody response by 1115-folds 2 dpc which was comparable to the 984-fold increase seen in the non-vaccinated challenged positive control group (G6) (P*<0.0001).

However, no other vaccinated challenged groups exhibited a similar response compared to the negative control group (Figure 5).

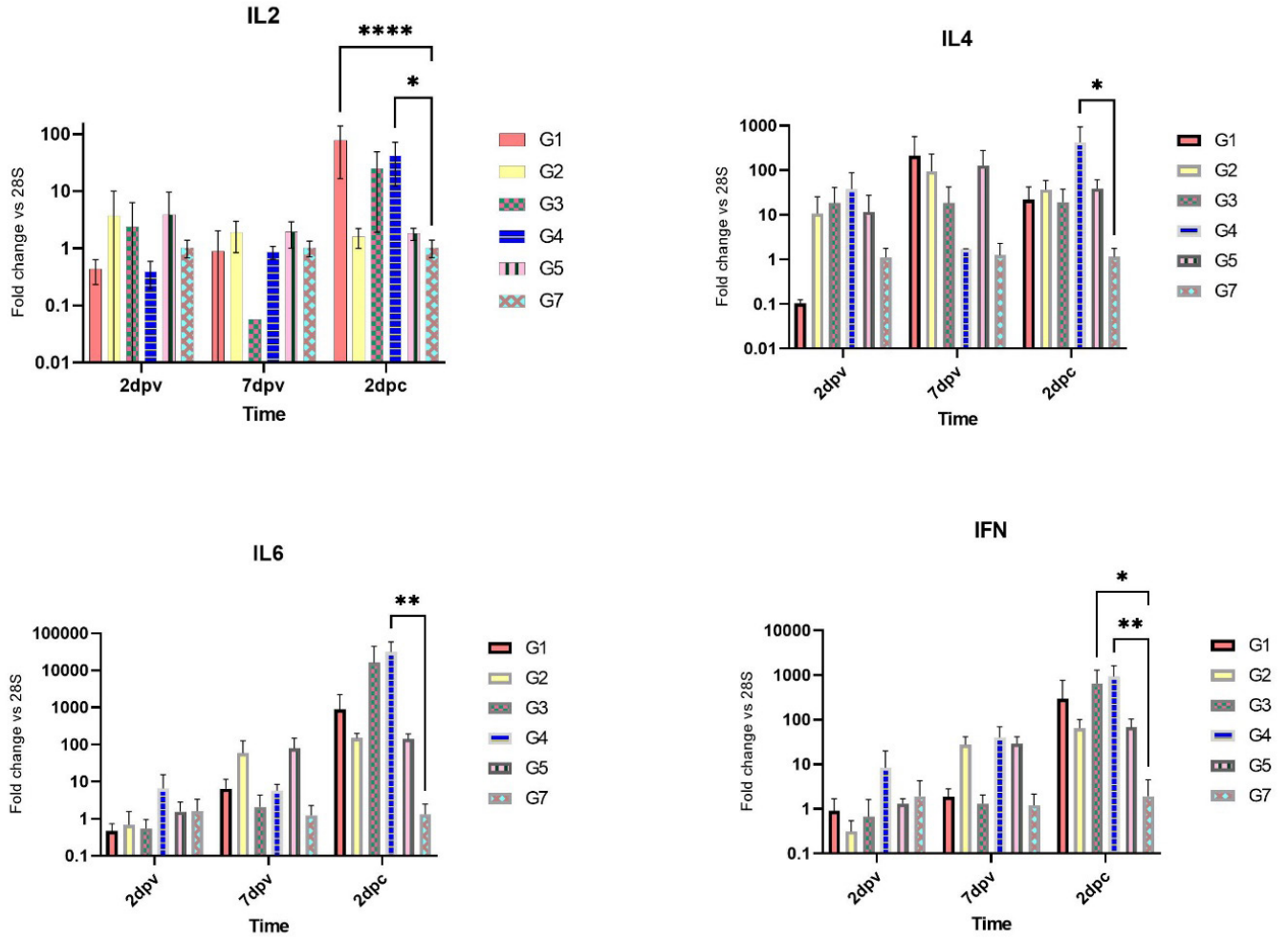

Figure 4: Cytokines foldchange expression levels in splenocytes of all chicken groups compared to 28S housekeeping gene (mean±STD).

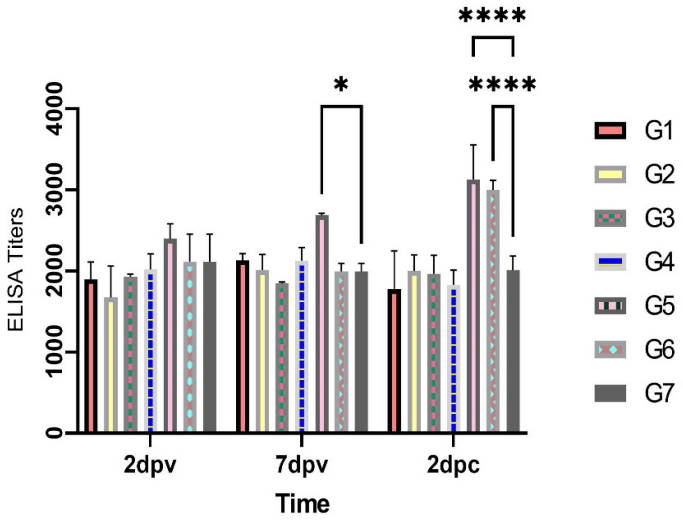

Figure 5: IgA ELISA titters in tracheal lavage for all chicken groups on intervening days after vaccination and challenge

HPAI is an extremely contagious avian illness that presents significant economic risks to the poultry industry and potential threats to public health (Neumann, 2015). The key to preventing avian influenza virus infection is vaccination. Current AI-inactivated vaccines are effective but the evolution of different AIV strains can reduce their efficacy. Determining the safety and effectiveness of vaccines is essential to identify the best ones. Progress has been made in developing vaccine antigens with adjuvants that act on mucosal surfaces to enhance cellular immunity, delivering a safer and more effective vaccine across the mucosa to mimic the natural route of infection and provide optimal protection (El Naggar *et al*., 2017). Nowadays, polymers and nanoparticles adjuvanted vaccines are more effective against viral disease infections (Corbanie *et al*., 2007; Shakya and Nandakumar, 2013). In this study we have tried to prove concept of mucosal adjuvants to enhance the immune response when used as inactivated vaccine excipient. In this regard, we formulated two novel mucosal monovalent AIV H5N8 inactivated vaccines using nanoparticles (IMS1313), and polymers (GEL01) adjuvants, and experimentally evaluate their role when applied via intranasal instillation and intramuscular injection methods compared

to MONTANIDE™ ISA70, on the vaccine potency and efficacy.

Our results documented the effects of MONTANIDE™ adjuvants on both innate and adaptive immune responses in immunized birds. In this regard, we used the HI test to determine the humoral specific immune response. HI results post-vaccination showed that the polymeric adjuvant vaccine GEL01/IM (G2) and ISA70/IM (G5) were more capable of conferring higher levels of specific HI antibodies above 6 Log^2, after 3 and 4 weeks of vaccination and reached the maximum (8.5 Log^2) with lower standard deviation coefficient (±0.9) where the variation between two groups did not exceed than 0.7 confirming their potency. Whereas groups vaccinated intranasally with either polymer GEL01/IN (G1) or nanoparticle IMS1313/IN (G3) gave a lower antibody response during the four weeks following vaccination that did not exceed 2.2 Log^2. However, we have used same antigen concentration /dose/vaccine type. Our findings align with those of (Joo *et al*., 2010) who observed that IgG production was stronger after intramuscular vaccination of mice compared to intranasal vaccination with inactivated avian influenza vaccine. They attributed this to the greater numbers of IgG antibody-secreting cells in the bone marrow and IgG memory B cells. Also coincided with those reported by (El Naggar *et al*., 2017) who found that SPF chicken groups vaccinated intranasally with MONTANIDE™ inactivated H9N2 IMS1313 or GEL01 showed weak HI titers (<5log2) throughout 15 weeks post-vaccination, while the MONTANIDE™ ESA71 intramuscularly vaccinated group maintained a higher titer (9log2) at 7 to 15 weeks after vaccination. Same as to the findings reported by (Ichinohe *et al*., 2006), who demonstrated that microparticle adjuvant resulted in lower HI levels than other adjuvants, also aligns with (Ismail *et al*., 2018) who confirmed the effectiveness of the inactivated AI ISA71 vaccine using inactivated H5N1 the MONTANIDE™ adjuvanted compared with IMS1313.

By highlighting how the immune system defends against AIV H5N8 infection and examining how our formulated vaccines help stimulate this defense mechanism using qRT-PCR, we identified and characterized the gene expression of cytokines IFN-γ, IL2, IL4, and IL6 in splenocytes after vaccination to understand their role in the immune response. Our results showed a numerical elevation in mRNA gene expression levels for cytokines were observed with GEL01/IM (G2) and ISA70/IM (G5), where IL-6 and INF-γ levels were increased at 7 dpv and IL-4 at 2 and 7 dpv. Our findings confirmed the participation and effect of vaccines in enhancing the cellular immune response, that in turn was reflected in an increase in the adaptive immune response after vaccination. Whereas IL-2 slightly increased at 2 dpv compared to the other vaccinated and negative control groups, these findings differ from those reported by (Li *et al*., 2023), where they observed that administering COS (Chitosan oligosaccharide), a biopolymer, successfully prevented the reduction in IL-2 protein and mRNA expression.

The data we have obtained is supported by the fact that cytokines play a crucial role in activating and regulating immune system cells. For example, IL-4 is considered a Th2 cytokine that stimulates humoral immunity by promoting the proliferation of activated B and T lymphocytes. This, in turn, leads to the differentiation of naive T helper cells into Th2 cells and the differentiation of B lymphocytes into plasma cells (Hershey *et al*., 1997). Interferon-γ, on the other hand, is classified as a Th1 cytokine that is involved in the induction of cell-mediated immunity and plays a vital role in activating macrophages and modulating the immune system. (Schoenborn and Wilson, 2007). Additionally, IL-6 is categorized as a proinflammatory cytokine that is important for the induction and regulation of the innate immune response, leading to the activation of B lymphocyte proliferation and supporting their growth, ultimately prompting the adaptive immune response (Ferguson-Smith *et al*., 1988; Tanaka *et al*., 2014a).

The results of our study differ from those of (Ismail *et al*., 2018) who observed a significant increase in gene expression levels of IL-6 and IFN-γ in SPF chickens following intraocular vaccination with H5N1 IMS1313 nanoparticles at two separate times. Additionally, (El Naggar *et al*., 2017) recorded higher levels of cytokines induced in SPF chickens immunized once with H9N2 IMS1313 or GEL01 administered intranasally and spraying for each type where spraying recorded higher levels. In our study, both polymers GEL01 and/or nanoparticles IMS1313.

IgA plays a crucial role in the mucosal immune system by enhancing the body's defense against infections when it is secreted. It is primarily found in mucous secretions, including those from the respiratory epithelium, genitourinary system, gastrointestinal tract, prostate, and sweat (Holmgren and Czerkinsky, 2005). We utilized ELISA to analyze the expression of IgA titers in tracheal lavage. Our findings demonstrate that only ISA70/IM G5 was able to effectively stimulate mucosal antibodies, leading to a notable increase in sIgA levels at 2dpv and a significant elevation after 7dpv ($P^*<0.05$) while other vaccinated groups did not show a difference compared to the negative control group. The findings in our study differ from those of (Tanaka *et al*., 2014b), who reported that live adjuvanted vaccination led to a significantly higher IgA titers response to booster vaccination compared to the control group. However, they are consistent with our results for the adjuvanted ISA70 IM vaccines. Also correspond with (Jang

et al., 2011), who observed higher levels of secretory IgA antibodies in hens immunized with certain adjuvants compared to those immunized with antigen alone. Specifically, profilin combined with ISA 71 adjuvants enhanced antibody levels more effectively than profilin alone, especially at 7 and 10 days after secondary immunization.

In the challenge experiment with HPAI H5N8 virus, the GEL01/IM) G2 and ISA70/IM) G5 (groups were the only ones that showed no clinical signs of avian Influenza (AI) and achieved the highest levels of protection of 85% and 90%, respectively, as well as virus shedding in tracheal swabs reduced by 2.2 $\log^2$ in G2 and completely disappeared in G5 6dpc. In the same regard, ISA70/IM (G5(, resulted in a much higher IgA titer in tracheal lavage, effectively preventing infection and the spread of the virus in infected birds. This led to the birds being free of clinical signs and achieving the highest survival rate. These findings are consistent with another research on this topic (El Naggar *et al*., 2017; Ismail *et al*., 2018). Interestingly, the genes GEL01/IM (G2) and ISA70/IM (G5) showed increased expression levels of IL-4, IL-6, and IFN-γ at 2 days post-challenge. This suggests that the innate immune response assists in promoting and regulating specific adaptive immunity against infection. It's important to note the role of IL-2, which was increased at 2 days post vaccination only in G2 and G5. IL-2 is secreted shortly after splenocyte stimulation with Concanavalin A and is produced by activated CD4+T cells and CD8+T cells. IL-2 is a type of cytokine signaling molecule in the immune system that aids in natural defense against microbial infections, activates γδ T cells, and stimulates the development of helper, cytotoxic, and regulatory T cells (Arenas-Ramirez *et al*., 2015; Choi and Lillehoj, 2000; Liao *et al*., 2011; Stepaniak *et al*., 1999).

In contrast, the remaining vaccinated groups were unable to fend off the viral infection. The GEL01/IN G1 polymer provided 46% protection, while the IMS1313/IN G3 nanoparticles were not effective in providing an acceptable level of protection, only 15%, resulting in varying degrees of avian influenza manifestations. The superiority of GEL 01 can be attributed to its high viscosity and density, which leads to slow absorption and allows it to form a gelatinous substance at the administration site. This unique characteristic provides a depot effect and extends the duration of exposure of the antigen to the immune system. In contrast, IMS1313 with lower viscosity and density is quickly absorbed due to its hydrophilic nature. As a result, GEL 01 offers enhanced immunity and protection compared to IMS1313 (SEPPIC, 2012).

These results were inconsistent with some previous publications where sprayed H9N2 GEL01 polymer adjuvants gave the highest protection level with no shedding in oropharyngeal swabs compared to IMS1313 nanoparticles (El Naggar *et al*., 2017). Additionally, the H5N1 IMS1313 intraocular adjuvants resulted in a 45% level of protection with virus shedding till 14 days post challenge. On the contrary, when primed with IMS1313/intraocular vaccine followed by ISA71/IM dose, a 90% protection level was achieved after challenge (Ismail *et al*., 2018). Unfortunately, the nanoparticles mucosal vaccine IMS1313/IM G4 when applied parenterally like polymer (G2) did not provide any benefit. It resulted in 0% protection, leading to 100% mortality and displaying characteristic signs of the disease (Ismail *et al*., 2018). On the other hand, gene expression levels of IL-6, IL-4, IL-2, and IFN-γ significantly increased, particularly in G4, demonstrating differences of 30,947, 386.2, 40, and 860.4, respectively, compared to the elevation levels of the protected groups, confirming the severity of infection (Wang *et al*., 2020). Furthermore, a considerable increase in IgA levels was noted in G6 (non-vaccination challenge), possibly due to the immediate production of IL-6 in response to infection, stimulating B cell proliferation and IgA secretion, and playing a crucial role in mediating inflammation during illness (Tanaka *et al*., 2014a). In group 4, levels of IL-6 and IFN-γ reached 31,097 and 927.4, respectively, at 2 days post-challenge, followed by 16200 and 646.6 in group G3. These groups exhibited the least protective effects in the experiment.

CONCLUSIONS AND RECOMMENDATIONS

We conclude that the vaccine adjuvant quality plays the corner stoon in initiation, magnitude and longevity of the specific immune response against target disease. Both GEL01/IM and ISA70/IM have demonstrated robust potency and efficacy. However, the superiority of ISA70 but the mucosal GEL01 adjuvant still showing promising results and space of improvement is required for the inactivated vaccine manufacturing. To achieve the highest level of protection, further studies on dual vaccination strategies involving both intranasal and intramuscular administration of different adjuvanted polymer is needed for optimizing the vaccine's efficacy against HPAI.

Ethics Approval and Consent to Participate

The study protocol was approved by the ethical and research committee at the Faculty of Veterinary medicine Suez Canal University.

ACKNOWLEDGEMENTS

The authors are grateful to the Avian and Rabbit Medicine Department at the Suez Canal University in Ismailia, Egypt, and Middle East for Vaccines (MEVAC) Co. For their technical assistance from all the authors.

NOVELTY STATEMENT

The mucosal GEL01 adjuvant still showing promising results and space of improvement is required for the inactivated vaccine manufacturing.

AUTHOR'S CONTRIBUTION

All authors contributed to this work. I. Ali is contributor to the process and methodological processing, acquisition of data and resources, formal analysis and writing of the first draft. W. Kilany; M. Zain El-Abideen and W. Elfeil are responsible for the supervision of sampling, methodology, and conceptualization of data and resource acquisition, formal analysis, and review of the study. M. El Demerdash contributed to conceptualization, formal analysis, validation, resources, and review. H. Abdien is the senior supervisor of the study, directing the work, analyzing the results, and writing the final manuscript. All authors carefully reviewed the final version of the manuscript and gave their approval.

Conflict of interest

The authors have declared no conflict of interest.

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