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Broader Early Humoral Immunity with Multi-Clade H5 Avian Influenza Vaccine Compared to Mono-Clade Formulation

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Abstract | Highly pathogenic Avian influenza (HPAI) remains one of the most significant infectious diseases affecting the global poultry industry, causing severe economic losses and posing a continuous threat to animal and public health. Since 2006, HPAI viruses of the H5Nx subtype, particularly those of the goose/Guangdong (Gs/GD) lineage, have been the most significant circulating strains worldwide. Egypt has remained an endemic hotspot for HPAI since 2006, with H5N1 and H5N8 viruses of multiple clades co-circulating in poultry. Thus, the implementation of extensive vaccination campaigns in Egypt faces persistent challenges related to viral evolution, the emergence and re-emergence of new strains, antigenic drift, and reassortment. This study aimed to evaluate the early humoral immune responses induced by two newly formulated, inactivated oil-emulsion avian influenza vaccines containing immunostimulant agents: a mono-clade vaccine (MEFLUVAC™ H5NX2) and a multi-clade vaccine (MEFLUVAC™ H5NX3). Responses were assessed in chickens at two weeks post-vaccination. Thirty chickens were vaccinated intramuscularly at 14 days of age with a single 0.5 mL dose of one of the two vaccines. Serum samples were collected at 1- and 2-weeks post-vaccination (WPV). The humoral immune response was assessed by hemagglutination inhibition (HI) assay against a panel of genetically distinct H5 antigens, including H5N8 (clade 2.3.4.4b), H5N1 (clade 2.3.4.4b), GD H5N1, GD H5N3, and H5 viruses belonging to clades 2.2.1.1 and 2.2.1.2. At week 2, MEFLUVAC™ H5NX3 elicited an HI antibody titer of 8.2 ± 2.8 $\log_2$ against H5N1 (clade 2.2.1.1), while MEFLUVAC™ H5NX2 elicited a titer of 7.6 ± 3.1 $\log_2$ against H5N8 (clade 2.3.4.4b). MEFLUVAC™ H5NX3 elicited more extensive cross-reactive hemagglutination inhibition responses against both homologous and heterologous H5 antigens compared to MEFLUVAC™ H5NX2, with the exception of GD H5N1 and H5N3, where the mono-clade vaccine exhibited no seroconversion rate and a lack of significant serological cross-reactivity (0.8 $\log_2$) against H5N3 GD for the multi-clade vaccine. The results demonstrate that both vaccines can induce a humoral antibody response; however, MEFLUVAC™ H5NX3 provided broader antigenic coverage against the panel of H5 strain antigens tested.

Keywords | Avian influenza, H5Nx Viruses, H5N1 Clade 2.3.4.4b, Hemagglutination inhibition assay; H5 vaccines, Immunostimulants, Early immunization

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INTRODUCTION

The poultry industry is significantly impacted by avian influenza (AI), a viral disease that has substantial financial consequences. AIV is a member of the Genus *Orthomyxovirus*, Family *Orthomyxoviridae*. The AIV genome consists of negative-sense, single-stranded, segmented RNA.The hemagglutinin (HA) and neuraminidase (NA) surface proteins are used to classify AIVs into 19 HA and 11 NA subtypes (Davis, 2014; Graziosi *et al*., 2024; Kandeil *et al*., 2017; Naguib *et al*., 2019a). AI viruses are categorized into two groups based on their pathogenicity: Low-pathogenic avian influenza virus (LPAIV) and highly pathogenic avian influenza virus (HPAIV). In addition to H5N8, H5N6, and H5N2 subtypes, the HPAIVs of zoonotic significance belong to the H5 and H7 subtypes that pose a threat to the poultry industry and can cause up to 100% mortality in poultry (Alexander *et al*., 2007; Bialy and Shelton, 2020; Diab *et al*., 2019). Since the first major HPAI H5N1 outbreak on a goose farm in China in 1996, Gs/GD-lineage viruses have undergone continuous genetic and antigenic evolution. Based on the genetic divergence of the H5 hemagglutinin (HA) gene, these viruses are categorized into ten major clades, designated 0 through 9 (Donis *et al*., 2008). The global spread of HPAI since 2006 has had a profound impact on poultry production, international trade, and public health, with Egypt being one of the most affected countries due to its long-term endemicity (Kayali *et al*., 2014). Within this context, multiple genetically distinct H5 subtypes have been detected in Egypt over the past decade. In 2019, a novel reassortant HPAI H5N2 virus was identified in a duck farm in the Dakahlia governorate, carrying seven gene segments derived from HPAI H5N8 and a neuraminidase (N2) gene originating from LPAI H9N2 viruses (Elfeil *et al*., 2025a; Hagag *et al*., 2019b; Sultan *et al*., 2019). Recently, the HPAI H5N1 subtype of clade 2.3.4.4b was identified and genetically described as having its origins in Egypt around 2021–2022 (Mosaad *et al*., 2023b; Sultan *et al*., 2024). These findings highlight Egypt's role as a dynamic ecosystem for H5 virus evolution and emphasize the ongoing challenge of achieving effective and broad protective immunity. Control of avian influenza relies on integrated strategies including prevention, management, and eradication. Among these, vaccination remains a critical tool for mitigating disease impact by enhancing host immunity, limiting viral replication, and reducing environmental virus shedding (Ali *et al*., 2024; Swayne, 2009). Given the continuous antigenic evolution of H5 viruses under endemic conditions, evaluating the breadth and early onset of vaccine-induced humoral immunity is essential. Accordingly, this study aimed to evaluate the early humoral immune responses, at two weeks post-vaccination, elicited by two inactivated H5 avian influenza vaccines, Mono-clade MEFLUVAC™ H5NX2 and Multi-clade MEFLUVAC™ H5NX3, in chickens vaccinated at 14 days of age. Antibody responses were assessed against a panel of homologous and heterologous H5 antigens to determine the extent of heterologous immune coverage.

MATERIALS AND METHODS

Vaccines

Two inactivated avian influenza vaccines were used, both formulated with the same adjuvant and a proprietary blend of immunostimulant agents. The Bivalent vaccination (MEFLUVAC™ H5XN2, MEVAC, Egypt) contained two reassortant seed strains: the rgA/ME-2023/H5N1 (H5N1) clade 2.3.4.4b, and the rgA/ME-2023/H5N8 clade 2.3.4.4b. the Trivalent vaccination (MEFLUVAC™ H5NX3, MEVAC, Egypt) contained three reassortant seed strains: the RG/A/chicken/ME-2018/H5N8 clade 2.3.4.4b, and rgA/ME-2023/H5N1 (H5N1) from the clade 2.3.4.4b) in addition to the RG/A/Chicken/Egypt/me 1010/2016 (H5N1) from the clade 2.2.1.1).

Experimental design

Thirty (two-week-old) White Leghorn Specific-Pathogen-Free (SPF) chicks were used in the experiment. The chicks were housed in BSL3 isolators for the duration of the trial. All birds had *ad libitum* access to feed and water and were maintained under hygienic conditions to ensure their optimal health and performance during the study. Prior to the immunization phase, birds were confirmed seronegative for the diagnostic antigens employed (data not shown). Both vaccines were administered intramuscularly (0.5 mL/bird) using sterile injection instruments.

Thirty experimental birds were split into two groups: G1 (n=15) received the MEFLUVAC™ H5NX2 vaccine, and G2 (n=15) received the MEFLUVAC™ H5NX3 vaccine. Serum samples were collected at weeks 1 and 2 after vaccination (WPV), as shown in Figure 1.

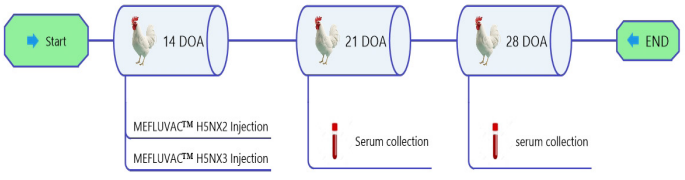

Figure 1: Schematic timeline of the experimental protocol. Group A was vaccinated with multi-clade trivalent MEFLUVAC™ H5NX3 at 14 days of age; meanwhile, Group B was vaccinated with mono-clade bivalent MEFLUVAC™ H5NX2. Each experimental bird received 0.5 mL via intramuscular injection. Serum collection was conducted at one- and two-weeks post-vaccination (WPV).

Hemagglutination inhibition (HI) test

The humoral antibody immune response after vaccination was evaluated using the HI test which is the standard test for evaluation of avian influenza seroconversion (WOAH, 2021). Diagnostic antigens for AIV H5 subtypes were provided by MEVAC, including viruses from clades 2.2.1.1. (H5N1), 2.2.1.2. (H5N1), H5N1 (clade 2.3.4.4b), and H5N8 2018 and 2023 (clade 2.3.4.4b). Furthermore, GD antigens (Royal GD Animal Health, Netherlands): H5N3 inactivated HI antigen (VLDIA 240 HAG lot NO. 21607-280721) and H5N1 inactivated HI antigen (VLDIA 330 HAG lot NO. 23601-240423).

Statistical analysis

Serological responses on day 14 post-vaccination (14DPV) were evaluated; data are displayed as arithmetic mean titers. One-way ANOVA, accompanied by Tukey's post-hoc contrasts, was employed to assess responses across diagnostic antigens within each vaccine. Unpaired t-tests, utilizing Welch's correction, were used to compare vaccines for each antigen. All tests were two-sided with $\alpha = 0.05$ following multiplicity adjustment.

RESULTS

Immune response in birds immunized with multi-clade vaccine MEFLUVAC™ H5NX3 against different AIV-H5 diagnostic antigens

Table 1 and Figure 2 summarize the hemagglutination inhibition (HI) antibody responses of chickens immunized with MEFLUVAC™ H5NX3 against a panel of heterologous AIV-H5 diagnostic antigens at 2-weeks post-vaccination (WPV).

At 1-week post-vaccination (W1), no detectable hemagglutination inhibition (HI) antibody titers were observed in any of the vaccinated birds against all tested H5 diagnostic antigens.

At 2 WPV, significant HI antibody titers (8.2 ± 2.8 log_2) were obtained by the vaccinated birds against H5N1 clade 2.2.1.1 (2016). Furthermore, potent immune responses were observed against H5N8 clade 2.3.4.4b (2018 and 2023), and H5N1 clade 2.3.4.4b (2023) with mean titers of (6.2 ± 3.1), (6.4 ± 3.1 log_2), and (5.5 ± 3.4 log_2), respectively. In the case of H5N1 clade 2.2.1.2 (2017), the antigen showed broad protective titers (3.4 ± 1.9 log_2). However, low titers against H5N3 GD (0.8 ± 0.9 log_2) were recorded.

Overall, while low or no reactivity was observed against genetically distant GD strains, MEFLUVAC™ H5NX3 exhibited broad and robust serological cross-reactivity, particularly against contemporary H5N8 and H5N1 clade 2.3.4.4b and 2.2.1.2 viruses. Statistically significant changes ($p < 0.05$) between diagnostic antigens within the same sampling time are indicated by different superscript letters.

Table 1: Serological reaction of birds immunized with MEFLUVAC™ H5NX3 and MEFLUVAC™ H5NX2 against different AIV-H5 diagnostic antigens.

Diagnostic antigen(s)	Multi-clade: MEFLUVAC™ H5NX3		Mono-clade: MEFLUVAC™ H5NX2	
	1WPV		2WPV	
H5N1 2.2.1.1 (2016)	Nd	Nd	$^{A}8.2\pm2.8^{a}$	$^{B}3.2\pm1.1^{b}$
H5N1 2.2.1.2 (2017)	Nd	Nd	$^{C}3.4\pm1.9$	$^{B}2.2\pm1.1$
H5N8 2.3.4.4b (2018)	Nd	Nd	$^{AB}6.2\pm3.1$	$^{A}6.2\pm2.2$
H5N8 2.3.4.4b (2023)	Nd	Nd	$^{AB}6.4\pm3.1$	$^{A}7.6\pm3.1$
H5N1 2.3.4.4b (2023)	Nd	Nd	$^{BC}5.5\pm3.4$	$^{A}5.9\pm2.9$
H5N1 GD	Nd	Nd	$^{D}0.0$	$^{C}0.0$
H5N3 GD	Nd	Nd	$^{D}0.8\pm0.9^{a}$	$^{C}0.0^{b}$

Not detected, The significance of HI antibody titers (log_2, Mean±SD) in titers utilizing different diagnostic antigens within the same group is indicated by capital superscript letters (A, B, C) on the left side; conversely, significant differences between the two. experimental groups utilizing the same diagnostic antigen are denoted by lowercase superscript letters (a, b, c) on the right side ($p < 0.05$). Serological response against the multi-clade vaccine, utilizing different diagnostic antigens (A); Serological response against the mono-clade vaccine, utilizing different diagnostic antigens (B); Multiclade vs mono-clade, 2 weeks post-vaccination, utilizing the same diagnostic antigen (C).

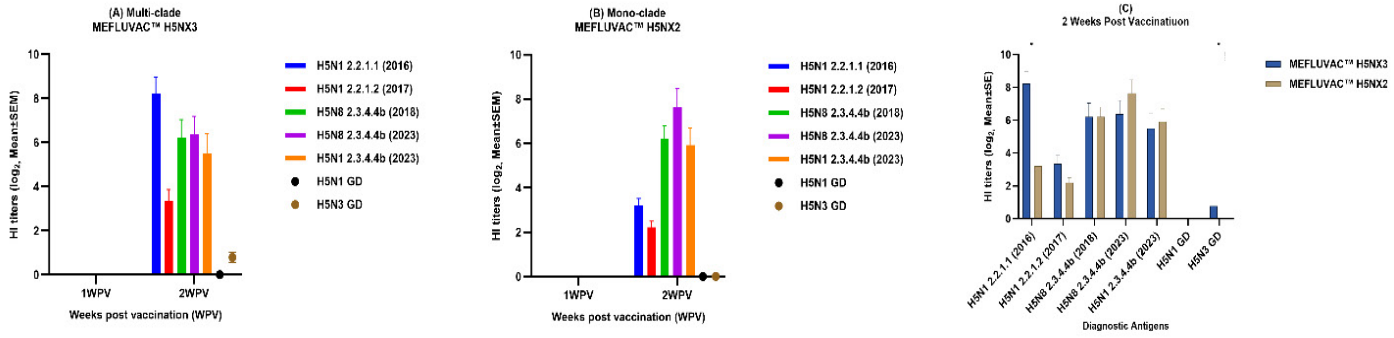

Figure 2: Hemagglutination inhibition (HI) antibody Mean and SE titers in birds immunized with MEFLUVAC™ H5NX2 and MEFLUVAC™ H5NX3 against different AIV-H5 diagnostic antigens.

Immune response in birds immunized with mono-clade vaccine MEFLUVAC™ H5NX2 against different AIV-H5 diagnostic antigens

The hemagglutination inhibition (HI) antibody responses of chickens vaccinated with MEFLUVAC™ H5NX2 against a panel of AIV-H5 diagnostic antigens at 2-weeks post-vaccination (WPV) are presented in Table 1, Figure 2.

At one-week post-vaccination (W1), None of the vaccinated birds had measurable hemagglutination inhibition (HI) antibody titers against any of the tested H5 diagnostic antigens.

At 2 WPV, HI antibody titers against H5N8 clade 2.3.4.4b were highest in vaccinated birds, especially against the 2023 isolate (7.6 ± 3.1 log_2) and the 2018 H5N8 strain (6.2 ± 2.2 log_2). In a similar vein, a significant cross-reactive reaction with a mean titer of 5.9 ± 2.9 log_2 was noted against H5N1 clade 2.3.4.4b (2023). On the other hand, lower antibody levels were found against the older Egyptian H5N1 clades 2.2.1.1 (2016) and 2.2.1.2 (2017), with mean titers of 2.2 ± 1.1 log_2 and 3.2 ± 1.1 log_2, respectively. At this moment, neither H5N1 GD nor H5N3 GD antibodies were found to be detectable.

Overall, MEFLUVAC™ H5NX2 elicited lesser immune responses against historical Egyptian H5N1 clades (2.2.1.x) and genetically distant GD strains, but it evoked a robust and widespread serological response against modern H5 viruses belonging to clade 2.3.4.4b, especially H5N8 strains.

Statistically significant differences ($p < 0.05$) between diagnostic antigens within the same sampling time are indicated by different superscript letters.

DISCUSSION

Avian influenza virus (AIV) is a major pathogen that affects many hosts and poses a serious threat to human and poultry health (Webby and Webster, 2001). The AIV infects a variety of wild and domesticated bird orders, such as Charadriiformes (e.g., gulls) and Anseriformes (e.g., ducks, swans, and geese) (Naguib *et al.*, 2019a). Additionally, based on its pathogenicity, AIV can be divided into HPAIV subtypes (H5 and H7) and LPAIV, which can mutate to generate HPAIV (Bonfanti *et al.*, 2014; Capua and Alexander, 2007; Deshpande *et al.*, 1987). A significant barrier to effective vaccination is the ongoing genetic and antigenic evolution of highly pathogenic avian influenza (HPAI) H5 viruses, especially in nations like Egypt, where the virus has become endemic. The emergence of several clades and reassortant genotypes, such as clades 2.2.1.x and, more recently, clade 2.3.4.4b viruses, after the 2006 introduction of H5 viruses in Egypt, have complicated the selection of vaccine strains and long-term control tactics (Hagag *et al.*, 2019a; Kayali *et al.*, 2014; Mosaad *et al.*, 2023b). The current study highlights the immunological benefits of multi-clade vaccine formulations by offering an early comparative evaluation of humoral immune responses elicited by a mono-clade vaccine (MEFLUVAC™ H5NX2) and a multi-clade vaccine (MEFLUVAC™ H5NX3). Both vaccines produced measurable HI antibody titers as early as two weeks post-vaccination, demonstrating that inactivated oil-emulsion H5 vaccines can elicit a rapid humoral immune response in SPF chickens. This early seroconversion is consistent with the known performance of oil-adjuvanted inactivated vaccines and is consistent with earlier results showing early seroconversion following inactivated avian influenza vaccination (Elfeil *et al.*, 2025a, b; Kilany *et al.*, 2014; Swayne, 2009). However, significant variations were noted between the two vaccination formulations in terms of the strength and range of antibody responses. Strong homologous and near-homologous HI responses were produced by the mono-clade vaccination MEFLUVAC™ H5NX2 against modern clade 2.3.4.4b viruses, especially H5N8 isolates from 2018 and 2023. These results highlight the significance of antigenic matching between circulating field viruses and vaccine strains, which has been consistently demonstrated to be essential for the best possible vaccine performance (Capua and Alexander, 2007; Donis *et al.*, 2008; Elfeil *et al.*, 2025a, b). The lower HI titers against the older Egyptian clades 2.2.1.1 and 2.2.1.2 suggest limited cross-reactivity, which most likely represent antigenic drift accumulated over time in the HA gene of H5 viruses circulating in Egypt (Naguib *et al.*, 2019b). The multi-clade vaccination MEFLUVAC™ H5NX3, on the other hand, produced more widespread and balanced HI antibody responses across several H5 clades. Strong responses against contemporary clade 2.3.4.4b viruses and significantly higher titers against the older H5N1 clade 2.2.1.1 demonstrate the added benefit of incorporating antigenically diverse seed strains in vaccine formulations. Previous research has shown similar results, with multi-strain or heterologous vaccine designs improving cross-clade immune recognition by focusing on conserved HA epitopes (Graziosi *et al.*, 2024; Sutton, 2018). The minimal or absent HI reactivity in both vaccine groups against the goose/Guangdong (GD) lineage H5N1 and H5N3 antigens is in keeping with previous research showing limited serological cross-reactivity between genetically and geographically distant H5 lineages (Elfeil *et al.*, 2025a, b; Naguib *et al.*, 2019b; Webby and Webster, 2001). However, compared to the mono-clade formulation, the identification of low-level HI activity against H5N3 GD in birds immunized with MEFLUVAC™ H5NX3 indicates a little extension in antigenic coverage. This

suggests that multi-clade vaccines may partially bridge antigenic gaps, even when full cross-protection is not achieved. Egypt's epidemiological context characterized by long-term endemicity, frequent reassortment, and repeated introduction of new variants highlights the limitations of mono-clade vaccination strategies (Hagag *et al*., 2019a; Mosaad *et al*., 2023a). Vaccines containing several antigenically different strains may provide more robust and adaptable immune protection in these situations. Multi-clade vaccinations may help minimize virus shedding, cut transmission rates, and mitigate vaccine escape variations by eliciting broader humoral responses, as previously proposed by (Graziosi *et al*., 2024; Swayne, 2009). Although multi-clade vaccinations have wider initial humoral responses, assertions that they may reduce virus shedding or transmission are conjectural and necessitate challenge studies for validation. Nevertheless, it is essential to recognize that the mono-clade vaccine generated robust HI titers against its corresponding contemporary H5N8 strains, underscoring the trade-off between specificity and breadth in vaccine development.

CONCLUSION AND RECOMMENDATIONS

In summary, while both vaccines elicited a humoral immune response, the multi-clade vaccine (MEFLUVAC™ H5NX3) provided broader cross-reactive antibody coverage against both historical and contemporary H5 viruses in this early assessment. These results support further investigation of multi-clade H5 vaccine formulations as a potentially more effective strategy for avian influenza control in regions like Egypt, where diverse H5 viruses co-circulate.

These findings support further investigation of multi-clade vaccine formulations as a potentially more effective strategy; however, conclusions are limited by the short timeframe of assessment and absence of challenge data.

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AUTHOR'S CONTRIBUTION

All authors contributed to this work. I.A. contributed to methodology, data acquisition, formal analysis, and writing the original draft. WHK, MZE, and WKE. Contributed to supervision, conceptualization, methodology, resources, analysis, and manuscript review. HMFA oversaw the study, directed the research, analyzed results, and wrote the final manuscript. All authors reviewed and approved the final manuscript.

Ethics approval

The Faculty of Veterinary Medicine at Suez Canal University granted ethical approval for this study.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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