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Protective and Cross-Protective Efficacies of Inactivated Recombinant H5N1 and H5N8 Vaccines Against Highly-Pathogenic Avian Influenza H5-Type Strains in Chickens

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Abstract

Background: Co-circulation of highly pathogenic avian influenza (HPAI) H5N1 and H5N8 viruses (clade 2.3.4.4b) threatens poultry production and public health, so vaccines that protect against both viruses are needed. This study evaluated the immunogenicity and cross-protective efficacy of inactivated recombinant (reverse genetics-derived) rgH5N1 and rgH5N8 vaccines ($10^{8.0}$ 50% embryo infectious dose [EID_{50}]/dose) against homologous and heterologous HPAI H5-type challenge in specific-pathogen-free (SPF) chickens. Vaccine strains were inactivated with 0.2% formalin and emulsified with Montanide ISA 71 VG adjuvant. SPF chickens (n = 10 per group) received rgH5N1 (G1), rgH5N8 (G2), or phosphate-buffered saline (G3, unvaccinated controls). Hemagglutination inhibition (HI) titers were measured at weeks 2, 3, and 4 post-vaccination. At week 4, birds were challenged intranasally with homologous or heterologous HPAI virus (10^6 EID_{50}/0.1 mL) and monitored for 10 days for clinical signs and mortality; viral shedding was assessed by real-time RT-PCR. Both vaccines induced HI responses that peaked at weeks 3–4. The rgH5N1 vaccine elicited high cross-reactive titers against H5N8 (9.2–9.4 log_2), whereas the rgH5N8 vaccine elicited lower cross-reactive titers against H5N1 (7.25 log_2). Both vaccines conferred 100% survival and no detectable viral shedding (cycle threshold [Ct] > 38) after homologous and heterologous challenge. All unvaccinated controls died, with high viral loads detected from day 3. Both inactivated vaccines provided complete cross-protection against heterologous H5-type challenge under controlled conditions, preventing mortality and detectable viral shedding. This supports their potential for controlling co-circulating H5N1 and H5N8 viruses in poultry, although confirmation in larger groups under field conditions is needed.

Keywords Avian influenza · H5N1 · H5N8 · Inactivated vaccine · Cross-protection · Poultry

Introduction

Highly pathogenic avian influenza (HPAI) H5Nx viruses remain among the most persistent and economically devastating threats to global poultry production and pose significant risks to public health. Since the emergence of the H5N1 Goose/Guangdong/96 lineage (Gs/GD) in China in 1996, continuous antigenic drift and genetic reassortment have resulted in the diversification of H5 viruses into multiple distinct clades and subclades, severely complicating disease control efforts worldwide [1–3]. Among these, the H5N8 virus (clade 2.3.4.4b) emerged as a particularly significant pathogen following reassortment events with low-pathogenic avian influenza viruses, causing unprecedented outbreaks associated with the culling of millions of poultry globally since its initial detection in 2014 [4, 5]. The concurrent circulation of H5N1 and H5N8 viruses—particularly within the same 2.3.4.4b clade—creates a complex epidemiological landscape in which further reassortment events could potentially yield novel strains with enhanced zoonotic potential and pandemic risk [6, 7].

Vaccination remains the primary strategic tool for controlling avian influenza in endemic regions; however, the efficacy of conventional inactivated vaccines is frequently compromised by antigenic mismatches between vaccine strains and circulating field viruses as a consequence of continuous antigenic drift [8, 9]. The development and evaluation of vaccines with broad, cross-protective immunity against heterologous strains is therefore critical for effective disease control. Recent advances in reverse genetics technology have enabled the rapid generation of recombinant (rg) vaccine strains more closely matching emerging field isolates [10]. Evidence demonstrates that rg-based H5N1 vaccines can offer substantial cross-protection against heterologous H5N8 viruses despite relatively modest cross-reactive antibody titers [4, 11]. For example, Ramírez-Martínez et al. [12] demonstrated that an inactivated H5N8 vaccine effectively protected both chickens and ducks against H5N1 challenge, with prime-boost vaccination completely suppressing viral shedding in chickens. Similarly, Maartens et al. [13] reported that a South African H5N1-based vaccine protected 100% of chickens against clinical disease and dramatically reduced viral shedding following challenge with an African strain of HPAI H5N8 (clade 2.3.4.4b).

The assessment of viral shedding as a measure of vaccine efficacy is increasingly recognized as critical for understanding vaccine potential to prevent silent virus circulation in vaccinated flocks and reduce transmission to naïve populations and wildlife [8, 14]. While clinical protection is essential, the induction of sterilizing or near-sterilizing immunity that prevents detectable viral replication is paramount for interrupting transmission cycles and achieving broader epidemiological control. Several studies have demonstrated that modern inactivated

vaccines can achieve such outcomes when antigenically well-matched to circulating strains [11–13, 15].

Despite these advances, the rapid evolution and continued co-circulation of H5Nx viruses necessitate ongoing evaluation of updated vaccine candidates against recent field isolates to ensure continued efficacy. To address this need, the present study aimed to evaluate the immunogenicity and cross-protective efficacy of newly formulated, inactivated recombinant rgH5N1 (clade 2.3.4.4b) and rgH5N8 (clade 2.3.4.4b) vaccines in specific-pathogen-free (SPF) chickens, against infections with homologous or heterologous HPAI H5-type strains of the same clade.

Materials and Methods

Viruses and Vaccine Preparation

The highly pathogenic avian influenza H5N1 (A/Muscovy duck/Egypt/PS21060OP/2023(H5N1)) and H5N8 (A/Poultry/Egypt/H5N8/2023) (both clade 2.3.4.4b) were used as vaccine candidate and challenge strains. The HPAI phenotype of both strains was confirmed by their intravenous pathogenicity index (IVPI): IVPI = 2.8 for H5N1 and IVPI = 2.7 for H5N8, both consistent with highly pathogenic classification [16]. The recombinant vaccine strains (rgH5N1 and rgH5N8) were kindly provided by the National Research Centre, Egypt. The rgH5N1 and rgH5N8 viruses were individually propagated in 10-day-old SPF embryonated chicken eggs for 72 hours at 37°C. Stock viruses were stored at −80°C until use.

Prior to vaccine preparation, viral titers were determined as the 50% embryo infectious dose (EID_{50}) using the Reed and Muench [17] method; stock titers were $10^{9.2}$ EID_{50}/mL for rgH5N1 and $10^{9.3}$ EID_{50}/mL for rgH5N8. Hemagglutinin (HA) titers were assessed by standard hemagglutination assay [18], yielding 512 HA units/50 µL for both stocks. The rgH5N1 and rgH5N8 viral stocks were completely inactivated using 0.2% formalin for 48 hours at room temperature. To confirm complete inactivation, 200 µL aliquots of each inactivated preparation were inoculated into the allantoic cavity of 10-day-old SPF embryonated chicken eggs (n = 5 eggs per passage), incubated for 72 hours at 37°C, and subjected to three successive blind passages; negative HA results and absence of viral RNA by RT-PCR at each passage confirmed complete inactivation. The inactivated antigens were subsequently emulsified with Montanide ISA 71 VG oil adjuvant (SEPPIC, Paris, France) at a ratio of 30% antigen to 70% adjuvant (v/v). Emulsification was performed on ice using a laboratory homogenizer for 10 minutes to

produce a stable water-in-oil (W/O) emulsion. Vaccines were stored at 4°C until use and emulsion stability was confirmed prior to vaccination.

Immunization of SPF Chickens and Serological Assessment

A total of thirty 21-day-old SPF White Leghorn chickens were randomly divided into three equal groups (G1, G2, and G3; n = 10 each). A single-dose vaccination regimen was used, consistent with initial immunogenicity screening studies and in accordance with the manufacturer's recommended dosing guidelines for this adjuvant formulation. Birds in G1 received 0.5 mL of the formulated rgH5N1 vaccine intramuscularly (IM) in the left breast muscle, while birds in G2 received 0.5 mL of the formulated rgH5N8 vaccine via the same route. Birds in G3 served as unvaccinated negative controls and received 0.5 mL of sterile phosphate-buffered saline (PBS; pH 7.2) IM. Blood samples were collected from all birds at weeks 2, 3, and 4 post-vaccination via brachial venipuncture. Sera were separated by centrifugation at 3,000 × g for 10 minutes and stored at −20°C until analysis.

Serological responses were assessed using the hemagglutination inhibition (HI) assay according to WHO guidelines [19]. Briefly, serum samples were treated with receptor-destroying enzyme (RDE) prior to testing. Serial two-fold dilutions were prepared in PBS, and each dilution (25 µL) was incubated with 25 µL of 4 hemagglutinin units (HAU) of either homologous (H5N1 or H5N8) or heterologous antigen for 30 minutes at room temperature. Following antigen incubation, 50 µL of 1% chicken red blood cells (RBCs) were added to each well and incubated for 30 minutes at room temperature. HI titers were calculated as the reciprocal of the highest serum dilution completely inhibiting hemagglutination and expressed as $\log_2$ values. Titers below 3 $\log_2$ were considered seronegative. All HI assays were performed in triplicate and data are presented as mean ± standard deviation (SD). Statistical comparisons of HI titers between vaccine groups at each timepoint and between homologous and heterologous antigen responses within each group were performed using the Mann-Whitney U test; $p < 0.05$ was considered statistically significant. Analyses were conducted using GraphPad Prism v.10.0 (GraphPad Software, San Diego, CA, USA).

Challenge Infection and Assessment of Vaccine Efficacy

At 4 weeks post-vaccination, vaccinated and control birds were subdivided for challenge studies. Group G1 (rgH5N1-vaccinated) was divided into two subgroups of 5 birds each: G1-A challenged with H5N1 (homologous) and G1-B challenged with H5N8 (heterologous). Group G2 (rgH5N8-vaccinated) was divided into G2-A (H5N8 homologous challenge) and G2-B (H5N1 heterologous challenge). Control group G3 was divided into G3-A (H5N1 challenge)

and G3-B (H5N8 challenge), 5 birds per subgroup. Challenge virus stocks (H5N1: $10^{7.8}$ EID_{50}/mL; H5N8: $10^{7.6}$ EID_{50}/mL) were diluted to achieve a challenge dose of 10^6 EID_{50}/0.1 mL per bird, administered intranasally by depositing 50 µL into each nostril using a sterile pipette under mild manual restraint. This challenge dose was selected to ensure 100% lethality in unvaccinated controls, consistent with standard HPAI challenge protocols [9], and represents a high-dose controlled exposure that may exceed natural field exposure levels.

Following challenge, all birds were monitored daily for clinical signs (depression, ruffled feathers, immobility, hemorrhages, neurological signs, and respiratory distress) and mortality for 10 consecutive days. Vaccine efficacy was calculated as: Protection (%) = (surviving birds at study termination / total challenged birds) × 100. For assessment of viral shedding, clonal and cloacal swabs were collected from each surviving bird on days 3, 6, and 9 post-challenge and placed in sterile tubes containing 1 mL of viral transport medium (VTM). Samples were stored at −80°C until analysis. Dead birds were swabbed immediately after death on the day of death.

Viral RNA was extracted from swab samples using the EasyPure® RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Real-time reverse transcription PCR (RT-PCR) targeting the influenza A matrix (M) gene was performed using the RealBest-Vet RNA Influenza A H5 detection kit (Vector-Best, Novosibirsk, Russia). PCR cycle threshold (Ct) values were recorded for each sample. Samples with Ct ≤ 38 were considered positive for viral RNA; samples with Ct > 38 or undetermined were considered below the limit of detection (negative). All samples were run in duplicate and mean Ct values are reported.

Ethics Approval

All animal experiments were conducted in accordance with institutional animal care and use guidelines and were approved by the Institutional Human and Animal Research Ethics Committee, Faculty of Veterinary Medicine, Beni-Suef University (Approval No.: PSAS-BSU-HAREC.026/02/018). The study design and procedures were reviewed and approved prior to initiation to ensure compliance with animal welfare standards.

Results

Antibody Responses Following Vaccination

Chickens vaccinated intramuscularly with the adjuvanted inactivated vaccines developed robust humoral immune responses over the 4-week post-vaccination period (Figs. 1 and 2). All

unvaccinated control birds (G3) remained seronegative throughout the pre-challenge period (HI titers < 3 $\log_2$ at all timepoints).

The rgH5N1 vaccine (G1) induced high HI titers against its homologous H5N1 antigen, with mean titers of 6.2 ± 0.4 $\log_2$ at week 2, 9.4 ± 0.3 $\log_2$ at week 3, and 9.5 ± 0.2 $\log_2$ at week 4 post-vaccination. Notably, G1 sera demonstrated significant cross-reactivity against the heterologous H5N8 antigen, with mean titers of 5.8 ± 0.5 $\log_2$ at week 2, 9.2 ± 0.3 $\log_2$ at week 3, and 9.4 ± 0.3 $\log_2$ at week 4 post-vaccination. Cross-reactive HI titers against H5N8 were not significantly different from homologous H5N1 titers at weeks 3 and 4 ($p = 0.21$ and $p = 0.18$, Mann-Whitney U test), indicating substantial cross-reactive potential.

The rgH5N8 vaccine (G2) elicited robust humoral responses against its homologous H5N8 antigen, with mean titers of 6.5 ± 0.4 $\log_2$ at week 2, 9.6 ± 0.2 $\log_2$ at week 3, and 9.7 ± 0.2 $\log_2$ at week 4 post-vaccination. However, cross-reactive HI titers against the heterologous H5N1 antigen were significantly lower, reaching 4.1 ± 0.6 $\log_2$ at week 2 and 7.25 ± 0.5 $\log_2$ at week 4 post-vaccination ($p = 0.004$ vs. homologous titers at week 4, Mann-Whitney U test). This asymmetric cross-reactivity pattern—with greater cross-reactivity of the rgH5N1 vaccine against H5N8 than vice versa—is discussed further below.

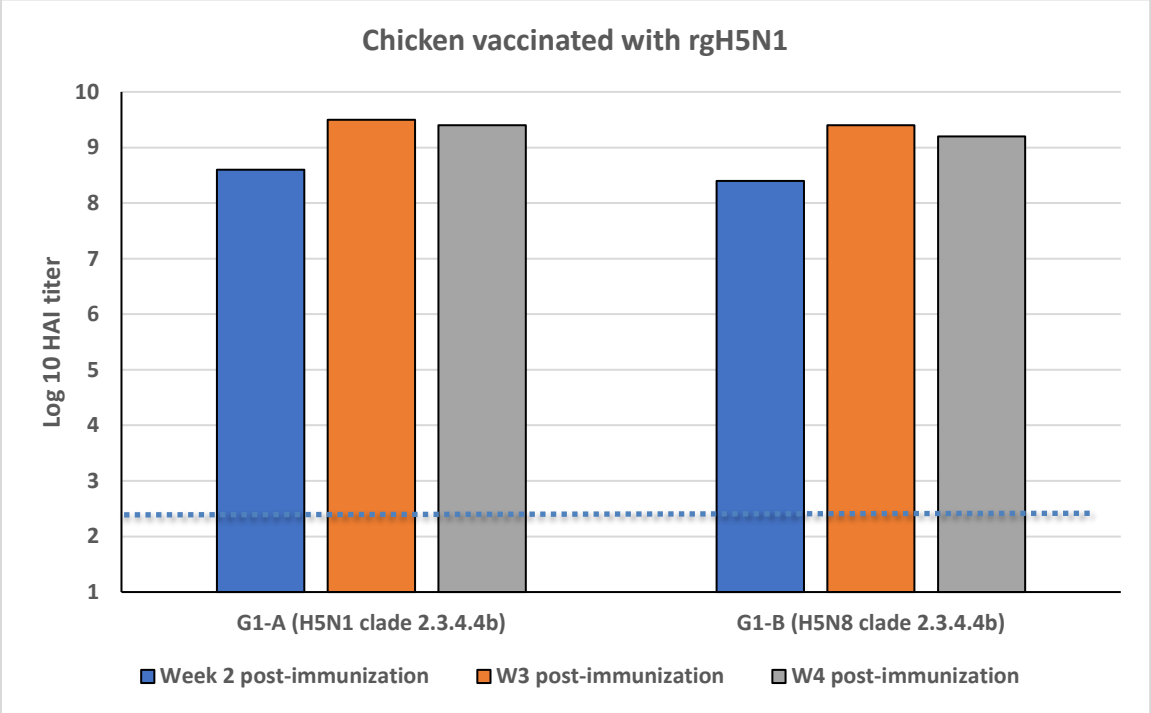


Fig. 1 Hemagglutination inhibition (HI) antibody titers (mean $\log_2$ ± SD) against homologous H5N1 (G1-A) and heterologous H5N8 (G1-B) antigens in chickens vaccinated with the inactivated rgH5N1 vaccine (G1) at weeks 2, 3, and 4 post-vaccination. Dashed line indicates the seropositive threshold (3 $\log_2$).

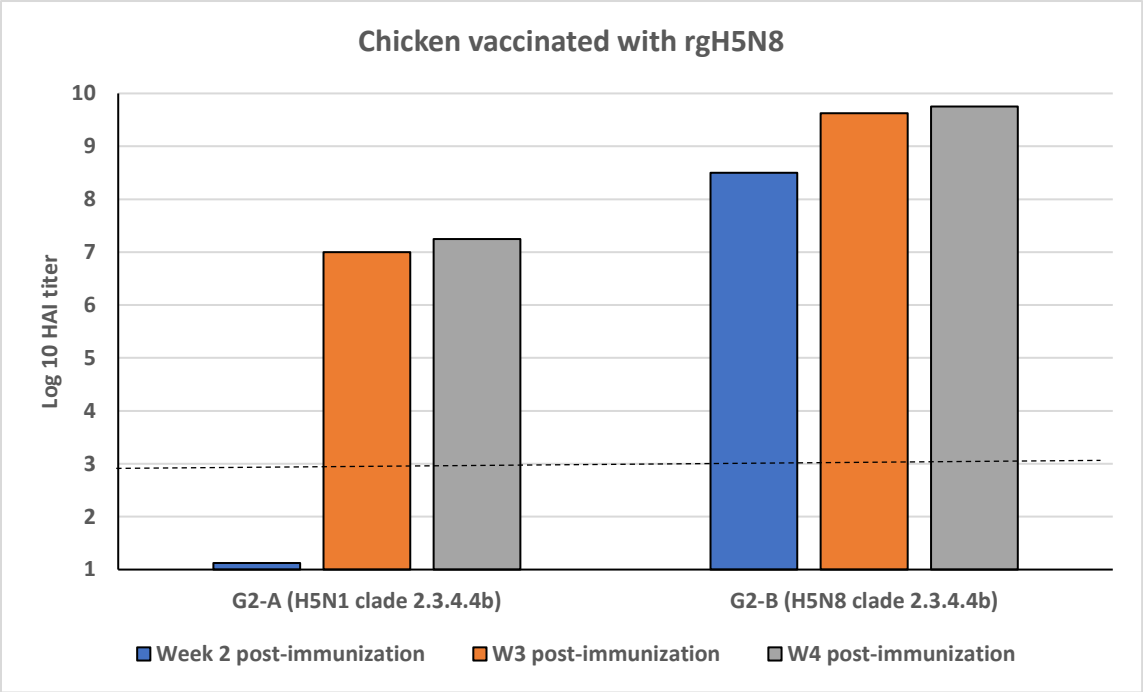


Fig. 2 Hemagglutination inhibition (HI) antibody titers (mean $\log_2 \pm$ SD) against homologous H5N8 (G2-B) and heterologous H5N1 (G2-A) antigens in chickens vaccinated with the inactivated rgH5N8 vaccine (G2) at weeks 2, 3, and 4 post-vaccination. Dashed line indicates the seropositive threshold (3 $\log_2$).

Clinical Protection and Survival Following Challenge

Following intranasal challenge at week 4 post-vaccination, both the rgH5N1 and rgH5N8 vaccines conferred complete clinical protection against homologous and heterologous HPAI H5-type viruses (Table 1). All rgH5N1-vaccinated birds achieved 100% survival when challenged with homologous H5N1 (G1-A: 5/5) and 100% survival when challenged with heterologous H5N8 (G1-B: 5/5). All rgH5N8-vaccinated birds similarly achieved 100% survival when challenged with homologous H5N8 (G2-A: 5/5) and 100% survival when challenged with heterologous H5N1 (G2-B: 5/5). None of the vaccinated birds displayed any observable clinical signs of disease throughout the entire 10-day monitoring period.

All unvaccinated control birds exhibited complete susceptibility. All H5N1-challenged controls (G3-A) began exhibiting clinical signs (depression, lethargy) by day 1 post-challenge, progressing to ruffled feathers, tremors, neurological signs, and respiratory distress by days 2–3, with complete mortality by day 5 (mean time to death [MTD] = 3.8 days). All H5N8-challenged controls (G3-B) first showed clinical signs on day 2, progressing similarly, with complete mortality by day 5 (MTD = 4.2 days). See Fig. 3 for clinical signs in control groups.



Fig. 3 Clinical signs in unvaccinated control chickens (G3) following challenge with H5N1 (G3-A) or H5N8 (G3-B)

Table 1 Clinical protection and survival rates among vaccinated and control groups following challenge with homologous and heterologous HPAI H5-type viruses. Numbers in mortality columns indicate deaths per day/total birds

Group	Subgroup (Challenge)	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	Protection (%)
G1 (rgH5N1)	G1-A (H5N1)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	100%
	G1-B (H5N8)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	100%
G2 (rgH5N8)	G2-A (H5N8)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	100%
	G2-B (H5N1)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	100%
G3 (PBS)	G3-A (H5N1)	2[a]	1[a]	2[a]	1[a]	—	—	—	—	—	—	0%
	G3-B (H5N8)	0[a]	2[a]	2[a]	1[a]	—	—	—	—	—	—	0%

[a] Indicates daily deaths; – indicates no surviving birds remaining from day 5 onward in control groups. All vaccinated birds showed no clinical signs through day 10 (study termination)

Viral Shedding in Vaccinated and Control Birds

Assessment of viral shedding via real-time RT-PCR of clonal and cloacal swab samples revealed striking differences between vaccinated and unvaccinated birds (Table 2). No vaccinated birds—regardless of vaccine group (G1 or G2) or challenge virus (homologous or heterologous)—yielded detectable viral RNA in either clonal or cloacal swabs at any of the three sampling timepoints (days 3, 6, and 9 post-challenge). All samples from vaccinated birds returned Ct values above the detection threshold (mean Ct > 38 or undetermined), indicating

that vaccination induced sterilizing or near-sterilizing immunity that prevented or reduced viral replication to below the assay's limit of detection.

In contrast, unvaccinated control birds (G3-A and G3-B) exhibited high levels of viral RNA in clonal swabs from day 3 post-challenge onward, with mean Ct values of 18.3 ± 1.2 (G3-A, H5N1) and 19.1 ± 1.4 (G3-B, H5N8) at day 3, consistent with active high-titer viral replication. Due to mortality of all control birds by day 5, swabs could only be collected from dead birds at day 6 (immediately post-mortem) and no day 9 samples were obtainable from control groups. Dead-bird swabs at day 5 (final mortality day) still yielded low Ct values (mean 22.4 ± 2.1), confirming persistent viral replication until death.

Table 2 Real-time RT-PCR results (mean Ct ± SD) from clonal and cloacal swabs in vaccinated and control birds following challenge

Group	Subgroup	Swab Type	Day 3 Mean Ct (±SD)	Day 6 Mean Ct (±SD)	Day 9 Mean Ct (±SD)	Detection Status
G1 (rgH5N1)	G1-A (H5N1)	clonal	> 38 (ND)	> 38 (ND)	> 38 (ND)	Negative
	G1-A (H5N1)	Cloacal	> 38 (ND)	> 38 (ND)	> 38 (ND)	Negative
	G1-B (H5N8)	clonal	> 38 (ND)	> 38 (ND)	> 38 (ND)	Negative
	G1-B (H5N8)	Cloacal	> 38 (ND)	> 38 (ND)	> 38 (ND)	Negative
G2 (rgH5N8)	G2-A (H5N8)	clonal	> 38 (ND)	> 38 (ND)	> 38 (ND)	Negative
	G2-A (H5N8)	Cloacal	> 38 (ND)	> 38 (ND)	> 38 (ND)	Negative
	G2-B (H5N1)	clonal	> 38 (ND)	> 38 (ND)	> 38 (ND)	Negative
	G2-B (H5N1)	Cloacal	> 38 (ND)	> 38 (ND)	> 38 (ND)	Negative
G3-A (PBS)	H5N1 challenge	clonal	18.3 ± 1.2	22.4 ± 2.1[a]	N/A[b]	Positive
G3-B (PBS)	H5N8 challenge	clonal	19.1 ± 1.4	23.1 ± 1.9[a]	N/A[b]	Positive

ND, not detected (below limit of detection; Ct > 38); positive, Ct ≤ 38. [a] Day 6 control samples collected from dead birds immediately post-mortem. [b] No day 9 control samples; all control birds died by day 5

Discussion

The emergence and rapid global dissemination of HPAI H5N1 and H5N8 viruses continue to pose unprecedented challenges for the poultry industry, with additional risk for zoonotic transmission underscored by recent sporadic human infections with H5N1 viruses [1, 7]. The potential for reassortment between co-circulating H5Nx strains further complicates control efforts. Vaccination remains the primary strategic approach for avian influenza control in endemic regions; however, continuous antigenic drift necessitates periodic evaluation of vaccine efficacy against novel circulating strains. This study demonstrates that newly formulated, inactivated rgH5N1 and rgH5N8 vaccines (clade 2.3.4.4b) can provide complete

clinical protection and suppress viral shedding to below the RT-PCR detection limit against both homologous and heterologous HPAI H5-type challenges in SPF chickens under controlled challenge conditions.

Our serological results demonstrate robust and cross-reactive humoral immune responses. The rgH5N1 vaccine induced high HI titers against both homologous H5N1 and heterologous H5N8 antigens (9.4–9.5 and 9.2–9.4 $\log_2$, respectively), with no statistically significant difference between homologous and heterologous titers at peak (weeks 3–4; $p > 0.05$). In contrast, the rgH5N8 vaccine showed strong homologous titers (9.6–9.7 $\log_2$) but significantly lower cross-reactive titers against H5N1 (7.25 $\log_2$ at week 4; $p = 0.004$). This asymmetric cross-reactivity pattern is consistent with the antigenic relationships described within clade 2.3.4.4b and aligns with previous observations by Mahmoud et al. [11] and Park et al. [4], and likely reflects directional antigenic differences between H5N1 and H5N8 HA proteins within this clade. It is important to note that HI titers, while a standard correlate of immunity, may not fully capture the breadth of protective immunity. Cell-mediated immunity, neuraminidase inhibition antibodies, and antibody-dependent cellular cytotoxicity (ADCC) likely contribute to the complete cross-protection observed, as suggested by the complete protection conferred by the rgH5N8 vaccine against H5N1 challenge despite relatively lower cross-reactive HI titers [9, 14]. Future studies should incorporate T-cell response assays and neuraminidase inhibition titrations to better characterize the breadth of protective immunity induced.

The prevention of detectable viral shedding in all vaccinated birds is a particularly significant finding for field applications, as it suggests these vaccines may prevent silent circulation of virus within vaccinated flocks and eliminate a major route of transmission to naïve birds, wildlife, and potentially humans. This observation is consistent with findings by Ramírez-Martínez et al. [12], who demonstrated that prime-boost vaccination with an inactivated H5N8 vaccine completely suppressed viral shedding in chickens following H5N1 challenge. Similarly, Maartens et al. [13] reported that an inactivated H5N1 vaccine reduced viral shedding to below detectability in 80% of birds after H5N8 challenge. The consistent prevention of detectable shedding across all subgroups in our study, using a single-dose regimen, provides additional support for the efficacy of these vaccine formulations. However, it is important to acknowledge that the RT-PCR detection limit (Ct threshold of 38) may not detect very low-level viral replication. Future studies should incorporate virus isolation from swab samples to confirm true sterilizing immunity versus near-sterilizing suppression below the PCR detection threshold.

Several factors likely contributed to the exceptional results observed under these controlled challenge conditions. First, the use of antigenically matched vaccine strains (both clade 2.3.4.4b, closely related to the challenge viruses) ensures optimal antigenic matching. Second, Montanide ISA 71 VG, a water-in-oil emulsion adjuvant, is well established for enhancing immunogenicity and promoting sustained antigen depot effects that favor both innate and adaptive immune responses [20]. Third, the complete inactivation process using 0.2% formalin, confirmed by triple blind passage with RT-PCR verification, ensures antigenic integrity without residual infectivity. However, several important limitations must be acknowledged. The use of SPF chickens eliminates confounding variables present in commercial flocks, including genetic heterogeneity, maternally derived antibodies, concurrent infections, and environmental stressors. Field conditions will typically involve lower and more heterogeneous challenge doses, variable immune status, and mixed-age flocks, which may yield different outcomes. The single-dose regimen employed here should be evaluated against prime-boost schedules, which may be needed in younger or immunologically naïve birds in field settings [12]. Furthermore, the challenge subgroup size of n = 5 birds, while consistent with initial efficacy screening studies and supported by power calculations for binary outcomes, limits statistical power for analyses beyond survival, particularly for quantitative shedding comparisons.

The cross-protective capacity of the rgH5N1 vaccine against heterologous H5N8 challenge, achieving both 100% survival and prevention of detectable shedding, is consistent with and extends findings from Zeng et al. [15], who demonstrated that H5N1 vaccines conferred full protection against lethal challenges with H5N2, H5N6, and H5N8 viruses with no detectable shedding in surviving birds. The observations of Park et al. [4] that H5N1 vaccines conferred 100% protection against H5N8 in mice and ferrets with markedly inhibited viral replication, despite low cross-reactive HI titers, further support the notion that mechanisms beyond HI antibodies—potentially including cytotoxic T lymphocyte responses targeting conserved internal proteins—contribute to cross-clade protection. The practical implication is that in regions where H5N1 and H5N8 viruses co-circulate, an rgH5N1 vaccine may provide dual protection, potentially simplifying vaccine programs, though continued surveillance and re-evaluation of antigenic match remain essential as H5Nx viruses continue to evolve.

Conclusion

The inactivated rgH5N1 and rgH5N8 vaccines (clade 2.3.4.4b) elicit robust, cross-protective immunity against homologous and heterologous HPAI H5-type viruses under controlled SPF challenge conditions, preventing mortality, clinical disease, and suppressing viral shedding to

below the RT-PCR detection limit. These findings highlight the potential of these updated vaccine candidates as tools for controlling the ongoing co-circulation of H5N1 and H5N8 viruses in poultry populations. Confirmatory field trials in commercial flocks, incorporating prime-boost regimens, larger group sizes, and assessment of cellular immune responses, are needed to validate these findings under real-world conditions. Ongoing surveillance of emerging H5Nx variants and periodic re-evaluation of vaccine efficacy remain essential components of a comprehensive H5 influenza control strategy.

Statements and Declarations

Funding

This study was funded by the Egyptian Company for Biological & Pharmaceutical Industries (Vaccine Valley), Egypt.

Competing interests

The authors declare no competing interest that could inappropriately bias or influence this work.

Ethics approval

All animal experiments were approved by the Institutional Human and Animal Research Ethics Committee, Faculty of Veterinary Medicine, Beni-Suef University (Approval No.: PSAS-BSU-HAREC.026/02/018).

Data availability

Data will be made available on request.

Author contributions

Mohamed N. F. Shaheen: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing – original draft. Adham Alkashef: Conceptualization, Investigation, Methodology, Formal analysis, Data curation. Vinayak Brahmakshatriya: Conceptualization, Methodology, Formal analysis, Data curation, Writing – review & editing. Hassan Abushanab: Conceptualization, Methodology, Investigation. Walid Hamdy Hassan: Conceptualization, data curation, Formal analysis, Writing – review & editing. All authors read and approved the final manuscript.

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