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RESEARCH ARTICLE

# Immunogenicity and Protective Efficacy of a Serum-Free Suspension MDCK Cell Culture-Based Inactivated H3N8 Avian Influenza Vaccine Compared with an Egg-Based Counterpart in Chickens

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**ABSTRACT**

H3N8 subtype avian influenza virus (AIV) circulates in poultry with occasional zoonotic potential and vaccination is crucial for preventing and controlling avian influenza. Here, two inactivated vaccines were prepared against the H3N8 AIV with GD1102 as vaccine strain: an embryonated chicken egg-based vaccine (E-vac) and a serum-free suspension MDCK cell-based vaccine (C-vac). The efficacy of both vaccines was evaluated in specific pathogen-free chickens. All vaccinated groups and the challenge control were intranasally challenged with $10^6$ $EID_{50}$ of GD1102 at 14 days post-vaccination (dpv) while the negative control group was left unchallenged. HI antibody titers, viral shedding, tissue viral loads, and pathological lesions were systematically assessed. Both vaccines elicited robust humoral immune responses at 7 dpv and the C-vac group had significantly higher HI titers than the E-vac group ($p$<0.01). However, at 14 dpv, both groups showed similar titers (HI>6 $\log_2$). Compared with the control group, both vaccines effectively reduced viral shedding and lessened histopathological lesions. These findings suggest that C-vac elicits a rapid antibody response and confers effective protection. Therefore, C-vac can be considered a promising new platform for H3N8 avian influenza vaccines.

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## INTRODUCTION

Avian influenza virus (AIV) inflicts substantial economic losses on the global poultry industry and constitutes a persistent zoonotic threat (Varvashenko *et al*., 2025). Multiple AIV subtypes, including H5N1, H5N6, H7N4, H7N9, H10N3, H10N8, and H3N8, possess pandemic potential and can infect humans, causing significant morbidity and mortality (Zhang *et al*., 2015; Huo *et al*., 2018; Naguib *et al*., 2019; Wang *et al*., 2021; Yang *et al*., 2022; Wei *et al*., 2025). The H3N8 influenza A virus primarily infects equines but has demonstrated cross-species transmission to various mammalian hosts, including canines, seals, swine, and humans (Solórzano *et al*., 2015; Hussein *et al.,* 2016; He *et al*., 2019; Zhou *et al*., 2021; Yassine and Smatti, 2022; Wasik *et al*., 2023). It also circulates among domestic poultry, waterfowl, and wildlife (Wang *et al*., 2023; Zhao *et al*., 2025; Mei *et al*., 2026). The positive rate for H3N8 in chicken flocks varies widely, ranging from 6.7% to 96.7% (Sit *et al*., 2022), reflecting extensive transmission and a substantial threat to the poultry industry. Since 2022, three laboratory-confirmed human H3N8 infections had been documented in China (Tan *et al*., 2022; Cheng *et al*., 2022; Sun *et al*., 2023), underscoring the urgent need for enhanced surveillance and preventive strategies.

Vaccination is the primary preventive measure against AIV. Conventionally, vaccines are produced using embryonated chicken egg-based techniques, which require a continuous egg supply (Ulmer *et al*., 2006) and are subjected to several constraints. During avian influenza outbreaks, egg supply may be disrupted; furthermore, embryonated eggs pose a risk of adventitious agent contamination. Compared with cell culture technology, the

egg-based approach is less defined, offers poor control over the substrate, shows batch-to-batch instability, and has limited large-scale production capacity. Suspension MDCK cells have been used to produce vaccines against certain AIVs, and these cell-based vaccines are at least as immunogenic as egg-derived ones (Ren *et al*., 2015; Gränicher *et al*., 2019).

However, few comparisons have been made between the embryonated chicken egg-based inactivated H3N8 vaccine (E-vac) and the serum-free suspension MDCK cell-derived inactivated vaccine (C-vac). Therefore, we designed this study to evaluate both platforms in SPF chickens for immunogenicity and protection. By monitoring antibody titers, viral shedding, tissue viral loads, and histopathological lesions, we aim to provide useful information for future H3N8 vaccine development.

## MATERIALS AND METHODS

**Virus:** A/chicken/Guangdong/1102/2022 (GD1102) was isolated from chickens on a commercial layer farm in Guangdong province, China, in November 2022. The subtype and genetic relationship of the isolate were confirmed by molecular and phylogenetic analysis against representative published novel H3N8 AIV sequences (Yang *et al*., 2022). The HA and NA genes of GD1102 clustered into the same phylogenetic branch as the reference strains and shared >98% nucleotide identity, which indicates that GD1102 is a novel H3N8 AIV. The virus was stored at the Diagnostic Center of Shandong Sinder Technology Co., Ltd. (Qingdao, China). The serum-free suspension MDCK cell-adapted GD1102 strain was provided by Shandong Sinder Animal Vaccine Co., Ltd. (Weifang, China).

**Vaccine preparation:** The $10^{th}$-passage embryonated chicken egg-derived GD1102 antigen and the $10^{th}$-passage serum-free suspension MDCK cell-derived GD1102 antigen were used for vaccine preparation. Both antigens had HA titers of 8 $\log_2$. Prior to inactivation and vaccine formulation, viral RNA was extracted from both antigen stocks, and the HA and NA genes were amplified by RT-PCR and sequenced to evaluate potential egg- or cell-adaptive mutations. Sequence comparison revealed no amino acid substitutions in HA and NA between either antigen stock or the parental GD1102 virus. Both antigens were inactivated with formaldehyde at a final concentration of 0.1% (vol/vol) at 4°C for 24 h. Post-inactivation HA titers remained at 8 $\log_2$. To verify complete inactivation, the inactivated antigens were inoculated into the allantoic cavity of 10-day-old SPF embryonated eggs (0.2 mL/embryo) and incubated at 37°C. After 72 h, undetectable HA activity in the allantoic fluid confirmed complete viral inactivation. A water-in-oil (W/O) emulsion vaccine was prepared by high-shear homogenization using Montanide™ ISA 71 VG adjuvant (SEPPIC, Paris, France) and inactivated antigen at a 7:3 (vol/vol) ratio. Briefly, the adjuvant was pre-stirred in a high-shear homogenizer at 6,000 × *g* for 1min, followed by gradual addition of the inactivated antigen. The mixture was then emulsified at 10,000 × *g* for 5 min. The emulsified vaccine was aliquoted into sterile vials, hermetically sealed, and stored at 4°C until use.

**Animal experimental design:** Four-week-old SPF White Leghorn chickens (Boehringer Ingelheim, Beijing, China) were randomly divided into four groups (n=20 per group).

1. E-vac group: chickens were immunized with the E-vac and subsequently challenged with the virus;
2. C-vac group: chickens were immunized with the C-vac and subsequently challenged with the virus;
3. Challenge control (CC) group: chickens were administered phosphate-buffered saline (PBS) as a mock vaccine and subjected to virus challenge;
4. Negative control (NC) group: non-immunized and unchallenged.

Vaccines were administered subcutaneously in the neck at a dose of 0.3mL per chicken. At 14 days post-vaccination (dpv), the C-vac, E-vac, and CC groups were challenged via the ocular-nasal route with $10^6$ $EID_{50}$/0.2 mL per chicken (Fig. 1). To comprehensively evaluate the immune protection efficacy, clinical observation, HI antibody responses, viral shedding, tissue viral loads, and histopathological examination were performed concurrently.

**Clinical observation:** Clinical signs were monitored using the scoring method described by Billington *et al.* (2025). Chickens were observed twice daily (morning and afternoon) and assigned scores according to a pre-defined clinical scoring scale (Table 1). Scores were defined as follows: 0=asymptomatic; 1=mild symptoms; 2=moderate symptoms and 3=severe symptoms.

**Table 1:** Total Clinical Scores of Each Clinical Sign Observed Following Challenge

| Clinical sign | Severity score | Group | | | |
|---|---|---|---|---|---|
| | | E-vac | C-vac | CC | NC |
| Depression | 1-3 | 10 | 8 | 43 | 0 |
| Greenish feces | 1-3 | 6 | 9 | 81 | 0 |
| Eyelid swelling | 1-3 | 14 | 8 | 74 | 0 |
| Dyspnea | 1-3 | 0 | 0 | 124 | 0 |
| Lacrimation | 1-3 | 0 | 0 | 48 | 0 |
| Total | / | 30 | 25 | 370 | 0 |

Clinical signs were graded as: 0, absent; 1, mild; 2, moderate; and 3, severe. The overall clinical score for each sign was determined by calculating the total scores recorded for all chickens in each group during the observation period. E-vac: embryonated chicken egg-based vaccine group; C-vac: cell-based vaccine group; CC: challenge control; NC: negative control.

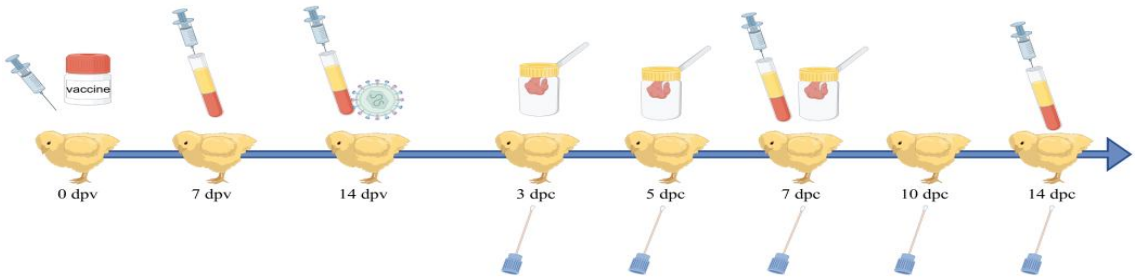


**Fig. 1:** Schematic diagram of vaccine efficacy evaluation of the H3N8 AIV vaccine. dpv: days post-vaccination; dpc: days post-challenge.

**HI antibody titer detection:** Serum was collected at 0, 7, and 14 dpv, and at 7 and 14 days post-challenge (dpc). HI assays were conducted using formaldehyde-inactivated embryonated chicken egg-based antigen as the test antigen following the WHO Manual on Animal Influenza Diagnosis.

**Quantification of viral shedding and tissue viral loads:** Oropharyngeal and cloacal swabs were collected at 3, 5, 7, 10, and 14 dpc. Meanwhile, three chickens per group were randomly selected and euthanized for tissue collection at the same time points. Tissue samples were minced and transferred into sterile centrifuge tubes containing PBS (pH 7.4) at a 1:3 (wt/vol) ratio. Samples were homogenized using a tissue grinder, subjected to 2–3 freeze-thaw cycles, and centrifuged sequentially at 4°C and 3,000 × *g*, 5,000 × *g*, and 8,000 × *g* for 5 min). For swab samples, cotton tips were placed into sterile tubes containing 2 mL PBS, vortexed thoroughly, and centrifuged at 4°C and 8,000 × *g* for 5 min. Resulting supernatants were used for nucleic acid extraction using a commercial DNA/RNA extraction kit (Vazyme, Nanjing, China) according to the manufacturer's instructions.

Virus was detected using an H3 RT-qPCR kit (Xinda Gene, Shandong, China) targeting the HA gene of H3 AIV to quantify viral copy numbers. The RT-qPCR cycling parameters were reverse transcription at 50°C for 5 min, initial denaturation at 95°C for 30 s, and then 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 30 s. Cycle threshold (Ct) values ≤36 were considered positive.

**Histopathological examination:** Lungs, trachea, and liver were collected at 3, 5, and 7 dpc and fixed in 10% neutral buffered formalin. Thirty-six chickens yielded 108 specimens for histopathological evaluation. Specimens were processed through dehydration, paraffin embedding, and sectioning at 5 μm (Leica RM2125RTS, Bensheim, Germany). Subsequently, sections were deparaffinized, rehydrated, and stained with H&E (Fischer *et al.*, 2008; Chen *et al.*, 2023). Microscopic examination was performed using an Olympus *CX41* fluorescence microscope (Olympus, Japan).

A semi-quantitative scoring system was applied to standardize lesion assessment: Grade 0: absent; Grade 1: focal or <25% tissue involvement (mild); Grade 2: multifocal or 25–50% involvement (moderate) and Grade 3: diffuse or >50% involvement (severe). Tracheal pathology was scored for epithelial integrity, ciliary preservation, and goblet cell hyperplasia. Pulmonary and hepatic pathology was scored for vascular congestion, hemorrhage, inflammatory infiltration, and parenchymal integrity.

**Statistical analyses:** All statistical analyses were performed using R version 4.3.1 (R Foundation for Statistical Computing, Vienna, Austria). HI antibody titers and viral loads are expressed as means ± SD. Differences among groups were assessed by one-way ANOVA with Tukey's post-hoc test for multiple comparisons. A $p<0.05$ was considered statistically significant.

## RESULTS

**Clinical signs and pathological changes:** No mortality occurred in any group during the 14 dpc observation period. Chickens in the CC group developed marked clinical signs, including depression, reduced feed intake, greenish diarrhea, and dyspnea with some individuals exhibiting eyelid swelling and lacrimation (Fig. 2A). In contrast, the C-vac and E-vac groups showed only mild, transient signs following challenge. At 3 dpc, three chickens from the CC group were euthanized for necropsy; gross lesions included mucoid exudate in the oropharynx (Fig. 2B), kidney enlargement (Fig. 2C), pulmonary congestion with focal consolidation (Fig. 2D), tracheal mucosal congestion and petechial hemorrhages (Fig. 2E), and thymic petechiae (Fig. 2F).

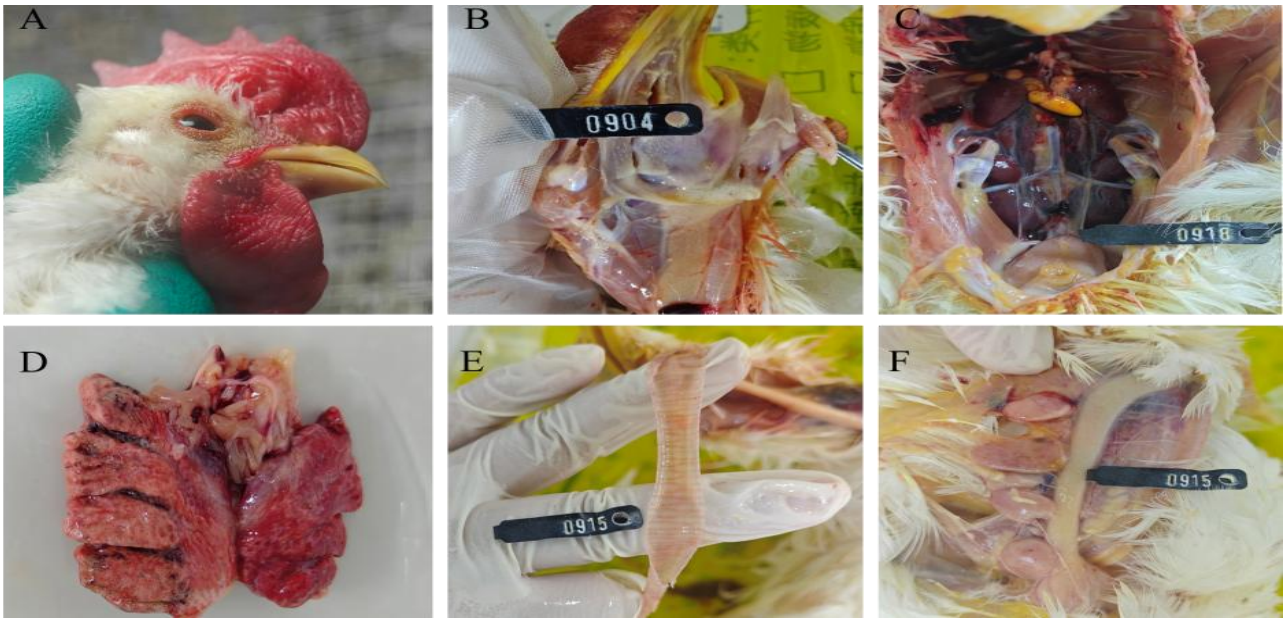


**Fig. 2:** Typical clinical manifestations and post-mortem lesions of SPF chickens infected with H3N8 AIV. A: eyelid swelling and lacrimation. B: mucoid exudate in the oropharynx. C: kidney enlargement. D: pulmonary congestion and focal consolidation. E: tracheal mucosal congestion with hyperemia and petechial hemorrhages. F: thymic petechiae.

**Humoral immune responses:** After vaccination, specific HI antibody responses were elicited in both the C-vac and E-vac groups, but not in the CC and NC groups. The C-vac group exhibited significantly higher HI titers than the E-vac group ($P<0.01$) and the CC group ($P<0.0001$) at 7 dpv. By 14 dpv, both vaccinated groups reached titers >6 $\log_2$, with comparable levels between them ($P>0.05$). Post-challenge, HI titers in both vaccinated groups remained significantly above those in the CC group at 7 and 14 dpc ($P<0.05$) (Fig. 3).

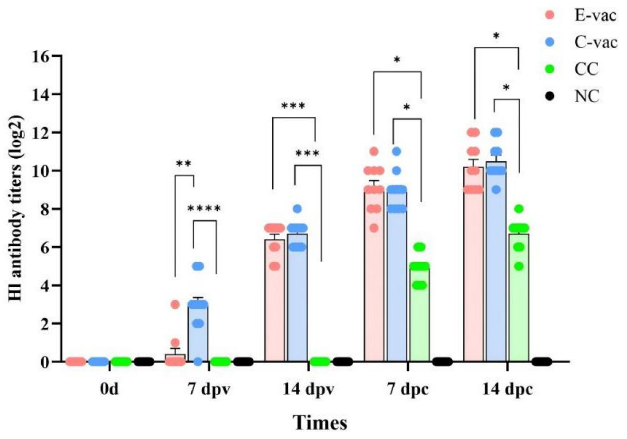


**Fig. 3:** Changes in serum HI antibody levels in SPF chicken post-vaccination and post-challenge. E-vac: embryonated chicken egg-based vaccine group; C-vac: cell-based vaccine group; CC: challenge control; NC: negative control; dpv: days post-vaccination; dpc: days post-challenge. (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

**Viral shedding:** To evaluate protective efficacy against viral shedding, oropharyngeal and cloacal swabs were collected and analyzed (Table 2 and Fig. 4). Following challenge, both vaccinated groups showed significantly lower viral loads in oropharyngeal swabs than the CC group at 3 dpc ($P<0.0001$) and 5 dpc ($P<0.01$; Fig. 4A). Accordingly, oropharyngeal shedding rates in vaccinated chickens ranged from 30–60%, substantially lower than the 100% shedding rate as observed in the CC group during this peak shedding period (Table 2).

**Table 2**: Viral Shedding Rates in SPF Chicken Post-Challenge

| Specimen type | Group | Viral shedding rates [% (no. positive/total)] | | | | |
|---|---|---|---|---|---|---|
| | | 3 dpc | 5 dpc | 7 dpc | 10 dpc | 14 dpc |
| Oropharyngeal swabs | E-vac | 60% (6/10) | 50% (5/10) | 10% (1/10) | 0% (0/10) | 0% (0/10) |
| | C-vac | 50% (5/10) | 30% (3/10) | 10% (1/10) | 0% (0/10) | 0% (0/10) |
| | CC | 100% (10/10) | 100% (10/10) | 40% (4/10) | 0% (0/10) | 0% (0/10) |
| | NC | 0% (0/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) |
| Cloacal swabs | E-vac | 10% (1/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) |
| | C-vac | 0% (0/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) |
| | CC | 80% (8/10) | 90% (9/10) | 60% (6/10) | 0% (0/10) | 0% (0/10) |
| | NC | 0% (0/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) |

E-vac: embryonated chicken egg-based vaccine group; C-vac: cell-based vaccine group; CC: challenge control; NC: negative control; dpc: days post-challenge.

Intestinal viral replication was effectively suppressed by both vaccines. No virus was detected in cloacal swabs from the C-vac group at any time point, whereas the E-vac group showed transient shedding in only 10% of chickens exclusively at 3 dpc (Fig. 4B, Table 2). By contrast, the CC group showed persistent cloacal shedding, with positive rates ranging from 60–90% between 3 and 7 dpc. Viral shedding had ceased in all groups by 10 dpc, while the NC group remained negative throughout the study.

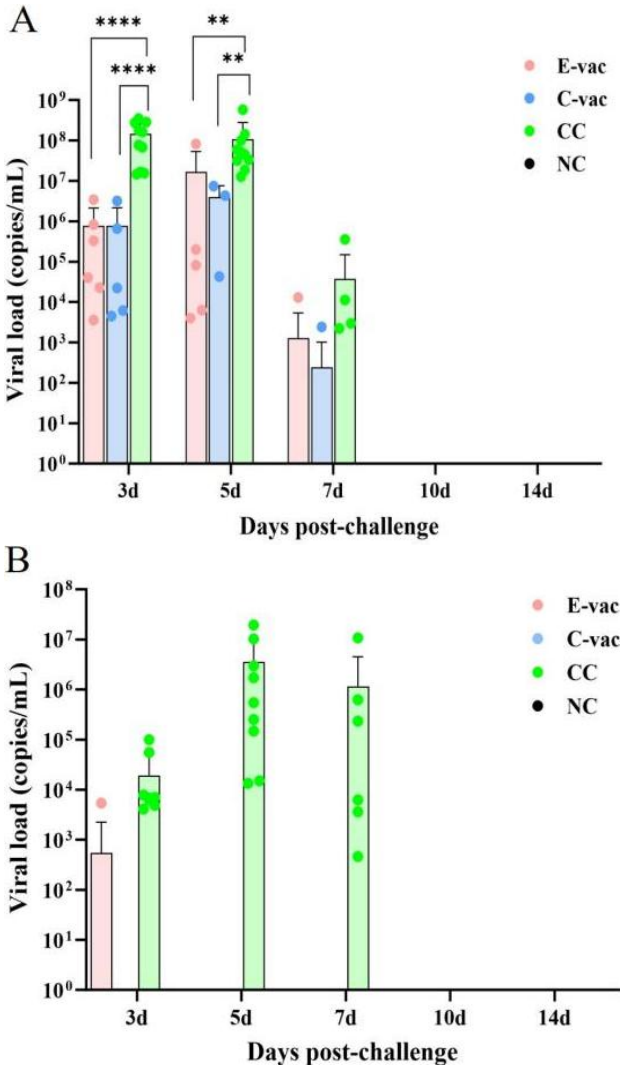


**Fig. 4:** Viral loads in oropharyngeal and cloacal swabs of SPF chicken post-challenge. (A): Oropharyngeal swabs; (B): Cloacal swabs. Group definitions as in Fig. 3.

**Tissue viral loads:** Tissue samples were collected from chickens in each group at 3, 5, and 7 dpc, and viral loads were determined by H3 RT-qPCR (Fig. 5). The results showed that high viral loads were detected in all tissues at 3 and 5 dpc, with peak levels in the trachea and lung in the CC group; and at 7 dpc, virus was restricted to the trachea, lung, ileum, spleen, and pancreas, and was undetectable in all other tissues. The C-vac and E-vac groups also showed detectable viral load in the trachea at all three time points; however, these levels were significantly lower than those in the CC group at 3 and 5 dpc. No virus was detected in the NC group at any time point.

**Histopathological findings:** SPF chickens in each group were euthanized and necropsied at 3, 5, and 7 dpc. Representative histopathological images in Fig. 6 and the descriptions below are derived from the 5 dpc time point, when lesions were most severe.

Lung tissues in both the E-vac and C-vac groups showed mild lesions, with focal hemorrhage in the secondary bronchi. In contrast, the CC group showed clear pathological

changes including indistinct alveolar capillary network, smooth-muscle hypertrophy/hyperplasia, severe vascular congestion, and inflammatory cell infiltration. The E-vac group showed mild goblet cell hyperplasia and some loss of cilia in the trachea, while the C-vac group exhibited only minor ciliary loss. In contrast, the CC group had severe goblet cell hyperplasia and extensive ciliary loss. As for the liver, both vaccinated groups had mild perivascular inflammatory cell infiltration, whereas the CC group showed severe infiltration. No histological damage was observed in the trachea, lung, or liver of the NC group.

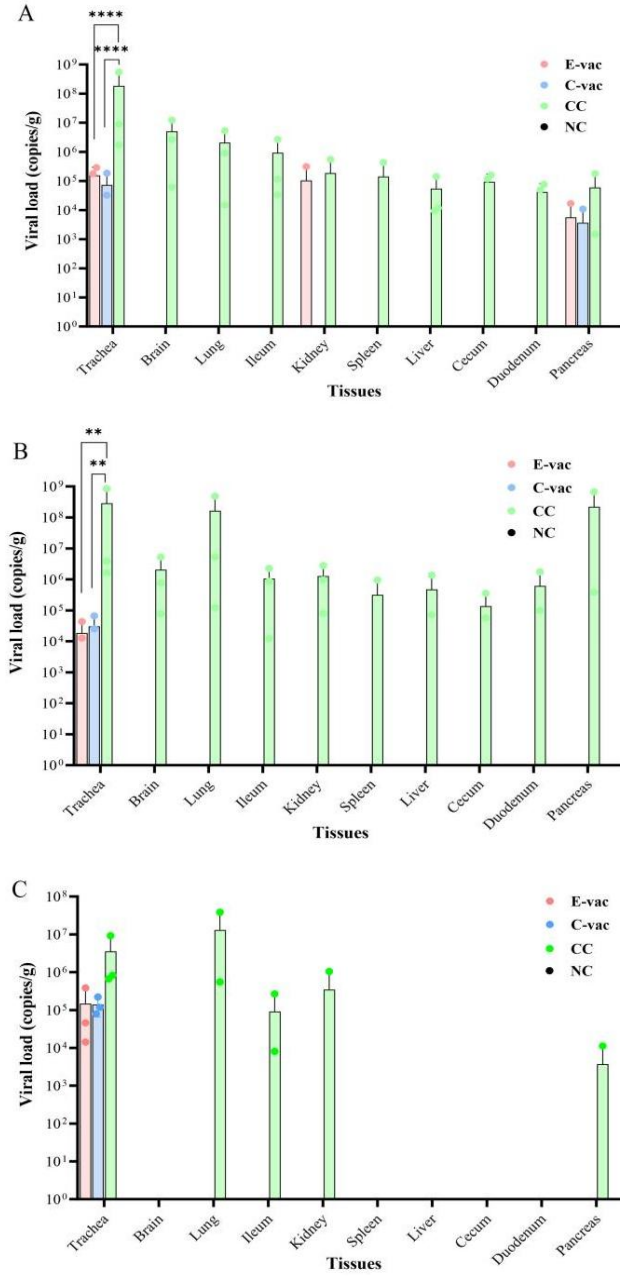


**Fig. 5:** Tissue viral loads of SPF chicken post-challenge. (A): 3 dpc; (B): 5 dpc; (C): 7 dpc. Group definitions as in Fig. 3.

## DISCUSSION

The emergence of H3N8 AIVs and their zoonotic potential poses a significant risk to the poultry industry and public health. Following the human cases in 2022, there is an urgent need for effective vaccines (Chen *et al*., 2023). In this study, we compared an egg-based and a cell-based inactivated vaccine against the novel H3N8 AIV. Our findings show that both vaccines offer protection, but the cell-based vaccine elicited a faster HI antibody response while providing similar protective efficacy. Both vaccines triggered a humoral immune response and protected chickens against challenge with GD1102. The vaccinated chickens showed lower viral loads, milder tissue damage, and much less viral shedding compared to non-vaccinated birds. Thus, inactivated vaccines can still prevent clinical disease and reduce viral replication in the trachea, lung and liver following H3N8 challenge. But the difference is in the rapidity of antibody appearance. The C-vac elicited a quicker humoral response and produced higher HI titers than the E-vac at 7 dpv. However, by 14 dpv, both groups reached similar HI titers (>6 $\log_2$, $P>0.05$). Antibodies control influenza infection by binding to viral antigens and blocking viral replication and shedding. A fast antibody response may limit viral replication and spread after challenge (Iwasaki and Pillai, 2014). N-glycosylation can affect the exposure of antigenic epitopes and the binding of HA and receptors (Schulze, 1997). Li *et al.* (2021) showed that HA proteins made in MDCK cells had different patterns of glycosylation (glycosylation site occupancy and glycan structure) compared to HA from eggs. That difference could affect how antibodies recognize the antigen. These altered glycans on cell-derived HA may help present immunogenic determinants better, which in turn activates B cells and speeds up antibody production.

A good vaccine should prevent or reduce clinical disease and lower viral shedding to limit the spread of infection among poultry flocks. In the CC group, all birds remained virus-positive at 5 dpc, whereas both vaccinated groups exhibited lower oropharyngeal viral loads at 3 and 5 dpc and a shorter shedding period. The C-vac reduced viral shedding from the upper respiratory tract more effectively than the E-vac. Given that AIVs spread mainly through the upper respiratory tract, early suppression of replication at this site is critical for lowering transmission risk. Tissue viral load analysis showed that both vaccines decreased virus levels in various organs compared with the CC group, indicating that vaccination also limited systemic viral replication after challenge.

Histopathological examination confirmed that both vaccines provided protection against H3N8 infection. The most severe lesions were observed at 5 dpc. In the CC group, chickens showed marked pulmonary vascular congestion and inflammatory cell infiltration, massive goblet cell hyperplasia, and ciliary destruction in the tracheal mucosa, and severe inflammatory infiltration in the liver. In contrast, both the E-vac and C-vac groups exhibited only focal inflammatory cell infiltration and relatively preserved tissue architecture. These findings indicate that vaccination not only reduced viral shedding but also lessened tissue damage following H3N8 infection.

The C-vac induced a rapid antibody response and reduced viral shedding and tissue viral loads, which suggests that it is a promising candidate for preventing H3N8 AIV. Our results support future efforts to develop MDCK cell–based inactivated vaccines as an alternative production platform for avian influenza vaccines.

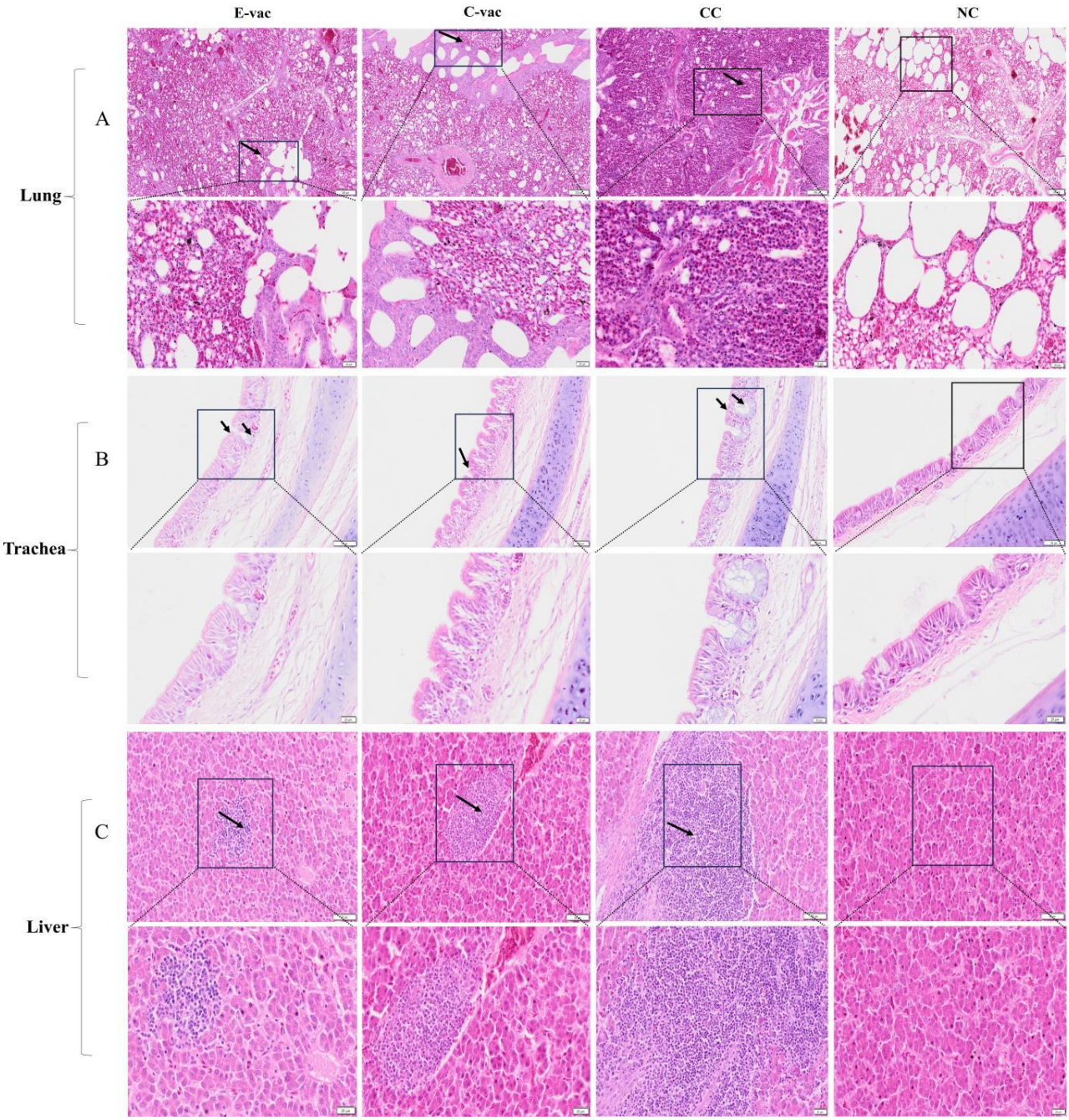


**Fig. 6:** Histopathological changes at 5 days post-challenge in SPF chicken infected with H3N8 AIV (H&E staining). (A) 200X magnification, scale bar=100μm; (B) and (C) 400X magnification, scale bar=50μm.

In the present study, we measured viral shedding and tissue viral loads using RT-qPCR, which can only detect viral RNA but cannot distinguish between infectious and non-infectious viral particles. Consequently, it remains unclear whether infectious viruses were present in the swabs and tissues. The observation period was not long enough to fully assess the long-term protective effects of C-vac. Future studies should perform virus isolation to confirm and quantify infectious virus and evaluate the strength and duration of immunity conferred by C-vac.

**Conclusions:** The MDCK cell-based inactivated H3N8 vaccine induced a rapid HI antibody response and provided protection against homologous H3N8 challenge. These results support the development of suspension cell culture-derived H3N8 AIV vaccines, which offer benefits for poultry health and public health preparedness.

**Author Contributions:** Conceptualization, Limin Li and Chunguo Liu; methodology, Xue Wang, Hao Shi, and Naijia Zhang; validation, Haiyan Zhang and Jialin Wang; formal analysis, Xue Wang; investigation, all authors; resources, Chunguo Liu and Limin Li; data curation, Naijia Zhang and Kai Yu; writing-original draft preparation, Xue Wang; writing-review and editing, Limin Li, Chunguo Liu, Abdul Khalique, and Muhammad Kashif Saleemi; visualization, Hao Zhu and Yongwei Zhao; supervision, Limin Li and Chunguo Liu; project administration, Chunguo Liu and Qingqing Song; Funding acquisition, Zhaoyang Li and Limin Li. All authors have read and agreed to the final version.

**Ethics Statement:** All animal experiments were conducted in accordance with the guidelines of the Animal Welfare and Ethics Committee of the Laboratory Animal Center, Hebei Agricultural University (Approval No. 2025099).

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