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Development of an inactivated H9N2 subtype avian influenza serological DIVA vaccine using the chimeric A/B NA epitope approach

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ABSTRACT

Vaccination is a critical strategy for controlling H9N2 avian influenza, a subtype with significant implications for poultry health and public safety. Current vaccines hinder serological differentiation between naturally infected and vaccinated animals, complicating disease surveillance and eradication efforts. Here, we developed a novel H9-subtype differentiating-infected-from-vaccinated-animals (DIVA) vaccine using reverse genetics. The recombinant virus, Re-H9-DIVA-J2, was engineered by replacing the neuraminidase (NA) gene of a clinically isolated H9 strain (A/chicken/Guangdong/J2/2016) with the NA ectodomain from a B/Yamagata-lineage influenza virus (B/Massachusetts/2/2012), while retaining six internal genes from the H1N1 PR8 strain. The chimeric virus exhibited low pathogenicity in chicken embryos, high growth titers (HA$\geq$8 $\log_2$), and stable genetic inheritance of the B-type NA marker over 10 passages. Three batches of inactivated vaccines were tested in specific-pathogen-free (SPF) chickens, demonstrating robust immunogenicity with hemagglutination inhibition (HI) antibody titers peaking at 10 $\log_2$ by 21 days post-vaccination. Challenge experiments confirmed full clinical protection and reduced viral shedding (above 90 % protection). Critically, sera from Re-H9-DIVA-J2-vaccinated chickens showed no cross-reactivity with A-type N2 protein in immunofluorescence (IFA) and ELISA assays, distinguishing them from sera of wild-type-infected or conventional H9N2-vaccinated animals. This study presents a safe, immunogenic H9 marker vaccine compatible with DIVA diagnostics, offering a promising tool for H9N2 control and eradication.

1. Introduction

Influenza viruses, classified into types A, B, and C, exhibit distinct biological and epidemiological characteristics (Jain et al., 2025). Influenza A viruses (IAVs) are the most diverse and impactful, capable of infecting a broad range of hosts, including birds, pigs, cattle, and humans (Martins et al., 2025; Tan et al., 2023; null et al., 2021). Their surface glycoproteins, hemagglutinin (HA) and NA, define viral subtypes and mediate critical functions: HA binds host cell receptors for entry, while NA cleaves receptors to facilitate viral release (Mitnaul et al., 2000; Wagner et al., 2002). To date, 18 HA (H1–H18) and 11 NA (N1–N11) subtypes have been identified in IAVs, forming combinations such as H1N1, H5N1, and H9N2 (Shao et al., 2017; Webster and Govorkova, 2014). This extensive diversity, coupled with a segmented genome of eight RNA segments (PB2, PB1, PA, HA, NP, NA, M, NS), enables frequent reassortment—a key driver of viral evolution and pandemic potential (Shao et al., 2017; Yamaji et al., 2025). In contrast, influenza B viruses (IBVs) are restricted to humans and seals, evolve more slowly, and are divided into two lineages (Victoria and Yamagata) (Ashraf et al., 2025, 2024; Tafalla et al., 2016). Influenza C viruses

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(ICVs) cause mild respiratory illness in humans and lack pandemic relevance (Sederdahl and Williams, 2020). Among these, IAVs—particularly the H9N2 subtype—pose a dual threat to animal and human health due to their zoonotic capacity and genetic plasticity (Song and Qin, 2020; Carnaccini and Perez, 2020; Pusch and Suarez, 2018).

The H9N2 subtype, a low-pathogenicity avian influenza virus (LPAIV), has become endemic in poultry across Asia, Africa, and the Middle East (Islam et al., 2025; Lee and Song, 2013; Sun and Liu, 2015; Sealy et al., 2018; Li et al., 2020). While it typically causes mild respiratory symptoms in birds, its silent spread facilitates persistent circulation and genetic exchange with high-pathogenicity strains (Wang et al., 2025; Peacock et al., 2019). Notably, H9N2 frequently donates its internal genes during co-infections, contributing to the emergence of novel viruses such as H5N1, H7N9, and H10N8 (He et al., 2025; Lam et al., 2015; Chen et al., 2014; Gao et al., 2013; Liu et al., 2003). This "genetic reservoir" role amplifies its public health risk, as evidenced by over 100 human infections since 1998, predominantly linked to poultry exposure (Song and Qin, 2020; Majumdar et al., 2025; Subbarao and Katz, 2000). Economically, H9N2 reduces egg production and increases susceptibility to secondary pathogens, costing the global poultry industry billions annually (Sims et al., 2020; Bonfante et al., 2018). Despite these threats, current control strategies remain inadequate (Carnaccini and Perez, 2020; Sealy et al., 2018; Dong et al., 2022).

Conventional inactivated vaccines can reduce clinical signs and, when well-matched, can mitigate viral shedding. However, they often fail to completely block transmission in field conditions and struggle to keep pace with antigenic drift (Parvin et al., 2020; Rahn et al., 2015; Liu et al., 2020). Moreover, the absence of a differentiating marker hinders serological surveillance in vaccinated populations, complicating eradication efforts in regions where vaccination is used (Suarez, 2012; Hasan et al., 2016; Alqazlan et al., 2022). To address this, DIVA strategies such as NS1-truncated vaccines or heterologous NA substitution have been explored (Nogales et al., 2022; Wang et al., 2011; Wu et al., 2009; Capua et al., 2003). NS1 truncation attenuates virulence but often compromises immunogenicity, while HA-NA mismatches in heterologous designs disrupt viral fitness. A promising alternative leverages reverse genetics—specifically the eight-plasmid system, which uses plasmids encoding each viral RNA segment to rescue recombinant viruses (Hoffmann et al., 2002, 2000; Lee et al., 2019). By modifying specific genes (e.g., replacing NA), this system enables precise engineering of chimeric viruses with tailored properties.

Here, we employed this approach to develop Re-H9-DIVA-J2, a novel H9N2 vaccine. We replaced the native NA ectodomain with that of a B/Yamagata-lineage virus (B/Massachusetts/2/2012), while retaining six internal genes from the H1N1 subtype A/Puerto Rico/8/1934 (PR8) strain—a backbone optimized for high-yield production in eggs. Crucially, preserving B-NA's packaging signals and transmembrane domains maintained HA-NA functional harmony, ensuring robust replication and genetic stability. The resulting chimeric virus combines the HA of a prevalent H9 strain (A/chicken/Guangdong/J2/2016) with a non-cross-reactive B-NA marker, enabling clear serological differentiation via N2-specific ELISA.

This innovation bridges the gap between vaccination and surveillance, offering a scalable solution for H9N2 control. By demonstrating the feasibility of A-B chimeras without compromising viral fitness, we redefine possibilities for influenza vaccine design. Future applications could extend this strategy to other subtypes, such as H5 or H7, where DIVA compatibility is equally critical. While challenges like long-term immunogenicity monitoring remain, this study provides a foundational framework for harmonizing disease control and diagnostics—a dual approach essential for mitigating avian influenza's global burden.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by the Experimental Animal Ethics Committee of Jiangsu Academy of Agricultural Sciences (Permit No. IACUC-AE-2024–01–022), which reviews protocols in accordance with Chinese national guidelines. The care and use of laboratory animals in this study were conducted in compliance with the principles outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) to ensure adherence to internationally recognized welfare standards. Viral propagation and titration were performed in a biosafety level 2 (BSL-2) facility at Zhejiang Difference Biotech Co., Ltd., while animal studies and serological testings were conducted in an animal biosafety level 2 (ABSL-2) facility at the Veterinary Research Institute of Jiangsu Academy of Agricultural Sciences.

2.2. Viruses and cells

Three H9N2 AIVs from diseased chickens, including A/chicken/Guangdong/J2/2016 (GD/J2/2016), A/Chicken/Zhejiang/ZJ02/2022 (ZJ02/2022) and A/Chicken/ Guangdong/GD03/2021 (GD03/2021). HEK-293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, USA) supplemented with 10 % fetal bovine serum (FBS; Gibical, USA) and 1 % penicillin-streptomycin (Thermo Fisher, USA) at 37°C under 5 % CO_2.

2.3. Plasmid construction

The HA gene from GD/J2/2016 was amplified by RT-PCR on an Eppendorf Mastercycler® nexus gradient system (Eppendorf SE, Germany) using primers flanking *BsmBI* restriction sites (Forward: 5'-CACACACGTCTCCGGGAGCAAAA GCAGGGGAATTTC-3' ; Reverse: 5'-CACACACGTCTCCTATTAGTAGAAACA AAGGGTGTTTTTGC-3'). Amplified HA (1.7 kb) was cloned into the pFlu vector to generate pFlu-H9J2-HA. A chimeric NA (PR8-BNA) was synthesized (Kingsray Biotech, China) by replacing the NA ectodomain of influenza A virus (H1N1 subtype) strain A/Puerto Rico/8/34 (PR8) with epitopes from IBV (Yamagata lineage) strain B/Massachusetts/2/2012. The chimeric NA gene was cloned into pFlu via *BsmBI* sites to generate pFlu-PR8-BNA. The six internal genes (PB2, PB1, PA, NP, M, NS) of PR8 were synthesized (Kingsray Biotech, China) and subcloned into pFlu.

2.4. Virus rescue

HEK-293T cells (70 % confluency) were co-transfected with 0.5 μg each of two plasmids (pFlu-H9J2-HA and pFlu-PR8-BNA) along with six PR8 internal gene plasmids, using Lipofectamine 3000 (Thermo Fisher Scientific, USA). At 24 h post-transfection, media were replaced with DMEM containing 0.5 μg/mL TPCK-trypsin (Sigma-Aldrich, USA). Supernatants were harvested after 48 h. Rescue of Re-J2-PR8BNA was confirmed by RT-PCR and Sanger sequencing (Genewiz, China).

2.5. Virus propagation and genetic stability

Re-H9-DIVA-J2 was serially passaged 10 times in 9-day-old SPF embryonated chicken eggs (2×10^4 PFU/egg) at 37°C. Viral titers were determined by HA assay using 1 % chicken erythrocytes. Genetic stability was assessed by amplifying the chimeric NA gene on an Eppendorf Mastercycler® nexus gradient system (Eppendorf SE, Germany) (forward primer: 5'-ATCTG CATTCACTAACCCCTG-3' ; reverse primer: 5'-TAGCACCTGGGTTCAGTCT-3') from passages P0, P5 and P10, followed by nucleotide sequencing (Genewiz, China). Sequence alignment was performed using DNASTAR Lasergene 17 (DNAStar Co., Madison, USA).

2.6. Serum preparation

NA (N2) Specific reference sera: Serum samples positive for H9N2 AIV (Re-2 strain), H5N1 AIV (Re-4, Re-5, and Re-6 strains), H7N9 AIV, Newcastle disease virus (NDV), and egg drop syndrome virus (EDSV) were obtained as reference materials from Qingdao Yibang Biotechnology Co., Ltd. (Qingdao, China).

Immune sera: Three-week-old SPF chickens (n = 5/group) purchased from Meiliya-Weitong Experimental Animal Technology Co., Ltd. (Beijing, China) were immunized subcutaneously with inactivated Re-H9-DIVA-J2, WJ57, GD/J2/2016, or HN03 vaccines (0.3 mL/dose) emulsified with a water-in-oil adjuvant (ExxonMobil). Booster doses were administered at 3-week intervals. Sera were collected at 7, 14, and 21 days post-immunization (dpi).

2.7. HA assay

HA assay for avian influenza H9 subtype was conducted following OIE standards (ANON, 2021). The viral antigen (H9 subtype) was provided by Harbin Weike Biotechnology Development Company. Briefly, two-fold serial dilutions of viral antigen were prepared in phosphate-buffered saline (PBS) using V-bottom 96-well microtiter plates. An equal volume of 1 % chicken erythrocyte suspension was then added to each well. The plates were incubated at 4℃ for 30–45 min until clear erythrocyte sedimentation patterns formed. The HA titer was determined as the reciprocal of the highest virus dilution causing complete hemagglutination, with one hemagglutination unit (HAU) defined as the minimal virus concentration required for complete agglutination.

2.8. HI assay

HI assay for avian influenza H9 subtype in chickens was performed according to OIE standards (ANON, 2021). The HI antigen (H9 subtype) and corresponding positive control serum were provided by Harbin Weike Biotechnology Development Company. Briefly, serum samples were subjected to two-fold serial dilutions in PBS, mixed with 4 HAU of viral antigen, and incubated at room temperature for 30 min. Subsequently, 1 % chicken erythrocyte suspension was added, and the mixture was incubated at 4℃ for 30–45 min. The HI titer was defined as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

2.9. IFA assay

The NA (N2) gene of H9 subtype influenza virus was cloned into the pCAGGS mammalian expression vector (Addgene #11160) using *KpnI* and *NheI* restriction sites (New England Biolabs, USA), generating the recombinant plasmid pCAGGS-N2. HEK-293T cells seeded in monolayer were transfected with 1 μg of pCAGGS-N2 plasmid using Lipofectamine 3000 (Thermo Fisher Scientific, USA), following the manufacturer's protocol. After 30 h of incubation at 37℃ under 5 % CO_2, cells were washed twice with PBS and fixed with pre-chilled methanol (-20℃) for 10 min. Fixed cells were blocked with 1 % bovine serum albumin (BSA; Sigma-Aldrich, USA) in PBS for 1 h at room temperature and subsequently incubated with chicken antisera (Re-H9-DIVA-J2 antiserum, H9N2 GD/J2/2016 strain antiserum, or H9N2-infected chicken serum) diluted 1:1000 in PBS for 1.5 h at 37℃. Following three washes with PBS, cells were incubated with Alexa Fluor 594-conjugated donkey anti-chicken IgY secondary antibody (1:200 dilution; Jackson ImmunoResearch, Cat. #703–585–155) for 1 h at 37 ℃. Fluorescence signals were visualized using an IX71 inverted fluorescence microscope (Olympus, Japan). lmages were captured using CellSens Dimension software (Olympus, Japan).

2.10. N2-specific indirect ELISA (N2-iELISA)

The N2 protein (Kingsray Biotech, China) was coated onto 96-well plates (l μg/well) at 37℃ for 1 h, followed by overnight incubation at 4℃. Serum samples were diluted 1:50 in PBS and incubated at 37℃ for 1 h, followed by incubation with HRP-conjugated sheep anti-chicken IgG (1:5000 dilution; Abcam, UK) for 1 h at 37℃. After washing, TMB substrate (Beyotime, China) was added and incubated for 15 min at room temperature. The reaction was stopped with 2 M H_2SO_4, and absorbance 450 nm (OD_{450}) was measured using a microplate reader (BioTek, USA). Results were calculated as signal-to-noise (S/N) ratios (sample OD / negative control OD), with samples considered positive when $S/N \geq 2.1$, suspicious when $1.5 < S/N < 2.1$, and negative when $S/N \leq 1.5$.

To assess the specificity of N2, sera positive for H5Nl, H7N9, NDV and EDSV, as well as positive control (H9N2 positive serum) and negative control (SPF chicken serum), were tested. Each serum sample was diluted at a predetermined ratio (1:50) and analyzed using the above established N2-iELISA. All samples were run in triplicate to evaluate potential cross-reactivity between the sera and the H9N2 subtype influenza virus NA (N2) protein (S1 Table).

2.11. Vaccination and challenge

The Re-H9-DIVA-J2 inactivated vaccine was prepared by treating virus-containing allantoic fluid (8.30 $\log_{10}$ EID_{50}/mL) with 0.1 % formalin (Sun et al., 2021; Ping et al., 2015), followed by emulsification with a water-in-oil adjuvant (ExxonMobil) at a 1:3 (vol/vol) ratio. The final formulation delivered a pre-inactivation antigen content of $10^{7.18}$ EID_{50} per 0.3 mL dose. This dosage, which is twice the minimum immunizing dose ($10^{6.88}$ EID_{50} in 0.15 mL) established in preliminary studies, was selected to ensure robust immunogenicity and protective efficacy while minimizing potential risks associated with antigen overloading, such as immune tolerance.

Three-week-old SPF White Leghorn chickens (Beijing Meiliya-Weitong Experimental Animal Technology Co., Ltd.) were randomly divided into three groups (n = 30/group) and vaccinated subcutaneously with 0.3 mL of Re-H9-DIVA-J2 vaccine, GD/J2/2016 vaccine or PBS. Serum samples were collected at 7, 14 and 2 l days post vaccination (dpv). At 21 dpv, chickens were challenged intravenously with 0.2 mL of one of the three H9N2 strains (GD/J2/2016, ZJ02/2022, or GD03/2021; n = 10 chickens per strain) containing $10^{7.5}$, $10^{6.5}$, and $10^{7.5}$ EID_{50}, respectively.

Pharyngeal and cloacal swabs were collected at 3, 5, and 7 days post challenge (dpc) and resuspended in 1 mL PBS, and titrated by inoculation into 10-day-old SPF embryonated eggs. Viral shedding was quantified via HA assay of allantoic fluid.

2.12. Statistical analysis

The HI antibody titers induced by Re-H9-DIVA-J2 and parent strain GD/J2/2016 were analyzed by unpaired student's two-sided t test. P values of 0.05 were regarded as showing statistically significant differences.

3. Results

3.1. Generation of the chimeric Re-H9-DIVA-J2 virus

To generate the DIVA-compatible H9N2 vaccine candidate, we employed an eight-plasmid reverse genetics system (Hoffmann et al., 2002; Lee et al., 2019). The HA gene of the isolated influenza GD/J2/2016 strain was amplified and sequenced. Phylogenetic analysis confirmed that the HA gene belongs to the same clade (h9.4.2.5) as the HA genes of H9 avian influenza viruses prevalent in China during 2018–2022, with sequence homology exceeding 91.3 %. (Fig. 1). The

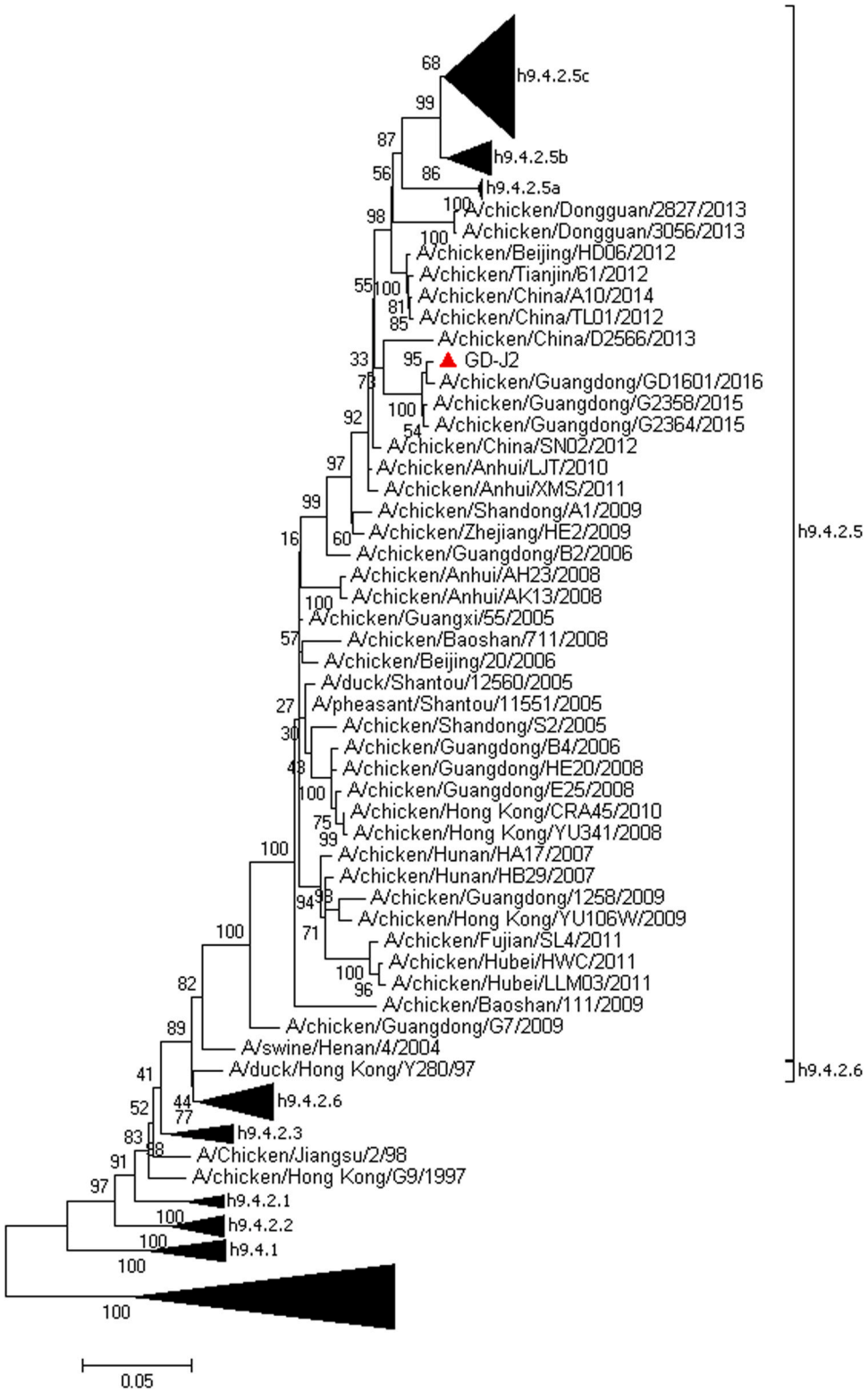

Fig. 1. Phylogenetic analysis of the HA gene from the H9N2 AIV GD/J2/2016 isolate. HA gene sequences of avian H9N2 influenza viruses were retrieved from the GISAID EpiCoV™ database (2016–2022). The phylogenetic tree was constructed by MEGA v7.0 with maximum likelihood (ML) method.

chimeric NA gene was synthesized to include the ectodomain of B/Yamagata-lineage NA (B/Massachusetts/2/2012) (Ping et al., 2016) fused to the transmembrane and cytoplasmic domains of PR8 NA, with packaging signals preserved (Fig. 2). Both genes were cloned into pFlu bidirectional plasmids. Co-transfection of 293 T cells with these plasmids and six PR8 internal gene plasmids (PB2, PB1, PA, NP, M, NS) yielded viable virus (Re-H9-DIVA-J2) after 48 h, confirmed by sequencing.

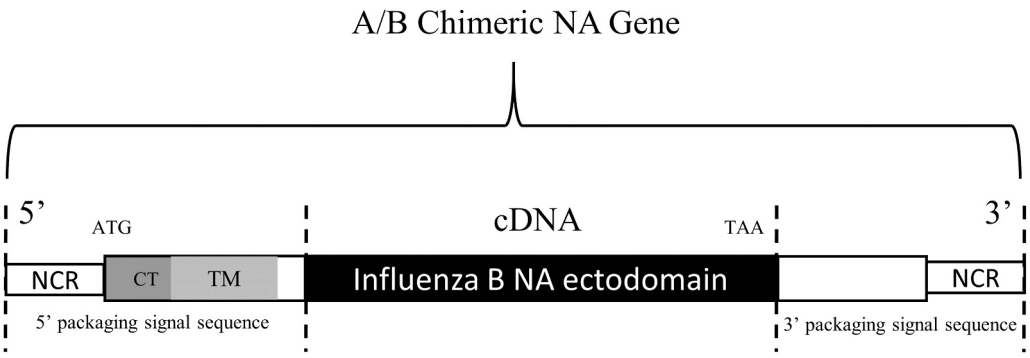

Fig. 2. Construction of a chimeric PR8-BNA gene through epitope replacement. The NA epitopes of the A/Puerto Rico/8/34 (H1N1; PR8) influenza virus were replaced with corresponding epitopes from the IBV strain B/Massachusetts/2/2012 to generate the chimeric PR8-BNA gene.

3.2. Recombinant virus proliferation and genetic stability in chicken embryos

The recombinant Re-H9-DIVA-J2 virus demonstrated efficient proliferation in SPF chicken embryos, with no mortality observed within 72 h post-inoculation. Serial passage in embryonated eggs confirmed its high-growth phenotype, yielding HA titers ≥ 8 $\log_2$ and EID_{50} titers ≥ 8.3 $\log_{10}$/mL across ten passages (Table 1). Notably, while the parental GD/J2/2016 virus also achieved high titers (HA 8–10 $\log_2$, EID_{50} 9.32–9.68 $\log_{10}$/mL over seven passages), it induced substantial embryo lethality (87 %–97 % initially, persisting at 89 %–95 %). In contrast, Re-H9-DIVA-J2 caused no embryo mortality (0 %; Tables 1 and S2). This indicates that the chimeric NA substitution retains high replicative capacity while significantly attenuating embryonic pathogenicity. Genetic stability analysis of the chimeric NA gene over these ten serial passages (P0 to P10) revealed only three silent nucleotide mutations (positions 196, 852 and 1269), with no amino acid changes (Fig. 3, Table 2). This conservation of the amino acid sequence strongly supports the structural and antigenic preservation of the B-type NA marker throughout passaging. Together with the consistent high-growth phenotype and low pathogenicity across all generations, these data demonstrate that Re-H9-DIVA-J2 exhibits the functional genetic stability necessary for a vaccine seed virus within a passage range relevant for manufacturing. These results establish that the recombinant virus combines robust proliferation with excellent genetic and phenotypic stability in eggs, confirming its compatibility with the H9 backbone and suitability for scaled vaccine production.

3.3. Immunogenicity and cross-protection against heterologous H9N2 strains

At 21 dpv, serum samples were collected from immunized chickens for quantitative assessment of H9N2-specific hemagglutination inhibition (HI) antibodies. The HI antibody titers induced by Re-H9-DIVA-J2 vaccine were similar to those induced by the parental strain GD/J2/2016 (Fig. 4), indicating that the chimeric substitution of PR8 (H1N1) -derived NA with B-type influenza virus NA epitopes preserved antigenic integrity and immunogenic potency.

To evaluate protective efficacy, chickens were challenged via the wing vein with the three circulating H9N2 avian influenza virus strains (GD/J2/2016, ZJ02/2022, and GD03/2021) in a 0.2 mL inoculum. The HA genes of these challenge strains share 89.2 %–93.5 % nucleotide homology, representing a moderate degree of antigenic divergence among recent field isolates (52 , 53). Despite this divergence, all chickens vaccinated with Re-H9-DIVA-J2 or GD/J2/2016 remained clinically healthy, showing no respiratory signs or mortality. Virological analysis revealed that 9 out of 10 vaccinated chickens in each group had no detectable viral shedding in pharyngeal and cloacal swabs throughout the 14-day monitoring period; one vaccinated chicken per group showed low-level shedding despite being clinically normal. In contrast, 100 % of PBS-control chickens demonstrated high-titer

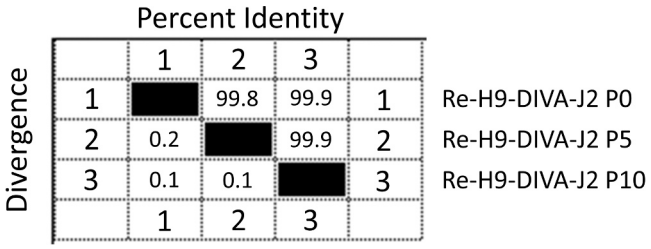

Fig. 3. Sequence alignment of the recombinant virus at passages P0, P5, and P10. The alignment reveals a high degree of sequence similarity among these generations, with only three nucleotide differences observed, all of which are silent mutations causing no amino acid changes. These results indicate strong genetic stability of Re-J2-PR8BNA, showing over 99.8 % sequence homology without insertions or deletions after ten passages in chicken embryos. Importantly, these minor changes did not enhance the virus's virulence or pathogenicity, supporting its potential as a safe vaccine candidate.

Table 2
Nucleotide sequence comparison of chimeric PR8-BNA gene across passages.

Mutation Site	Virus Generation		
	P0	P5	P10
196	G	A	A
852	A	T	C
1269	T	C	T

Table 1
Recombinant virus proliferation and genetic stability in chicken embryos.

Virus Generation	72-Hour Titer (HA potency, $\log_2$)	72-Hour Embryo-Lethal	EID_{50}/mL
P0	10	0	8.30
P1	10	0	8.50
P2	8	0	8.32
P3	10	0	8.38
P4	9	0	8.32
P5	10	0	8.68
P6	10	0	8.50
P7	9	0	8.38
P8	10	0	8.32
P9	9	0	8.68
P10	10	0	8.50

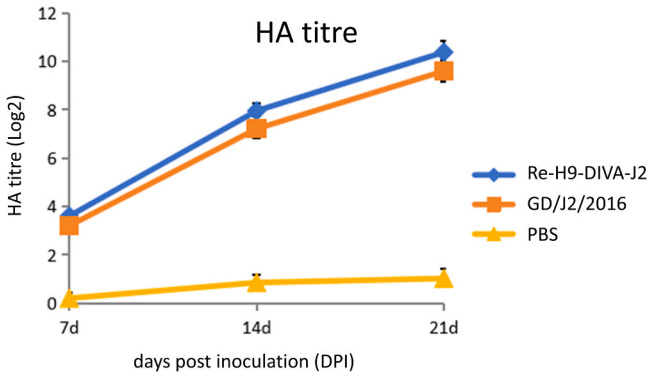

Fig. 4. HA titers were determined by HA assays. The values presented are the average of three independent experiments ± SD. P values were calculated by unpaired Student's two-sided t test. P values of 0.05 were regarded as showing statistically significant differences. Red and blue asterisks indicate the comparison of the Re-H9-DIVA-J2 or parental strain GD/J2/2016 with PBS.

shedding at 5 dpc, with persistent shedding until day 7. These findings confirm that Re-H9-DIVA-J2 provides cross-protective immunity equivalent to conventional H9N2 inactivated vaccines against heterologous H9N2 field strains.

3.4. DIVA capability validated by serological discrimination

IFA and N2-iELISA conclusively demonstrated the DIVA potential of Re-H9-DIVA-J2. IFA using 293 T cells expressing H9N2 N2 protein showed strong reactivity with sera from H9N2-infected or conventionally vaccinated chickens (Figs. 5A, 5B), but no reactivity with Re-H9-DIVA-J2 antiserum (Fig. 5C). Similarly, N2-iELISA confirmed that sera from Re-H9-DIVA-J2-vaccinated chickens remained negative (S/N < 1.5), while conventional vaccine and infection sera scored positive (S/N > 4.83; Table 3). This antigenic discrimination provides a dual advantage for field surveillance and outbreak containment.

To further confirm that the N2-iELISA could reliably discriminate vaccinated chickens from those experiencing breakthrough infections, sera from Re-H9-DIVA-J2-vaccinated chickens were tested at 21 dpc with GD/J2/2016. A majority of these chickens (9 out of 10) seroconverted, showing positive N2-iELISA results (S/N > 2.1; see Supplementary Table S3). This demonstrates that the companion diagnostic remains effective in detecting infection even within a vaccinated population, fulfilling a key requirement for practical field surveillance.

3.5. Safety and growth kinetics in SPF chickens

Safety evaluations confirmed no adverse effects on weight gain or clinical health in SPF chickens immunized with Re-H9-DIVA-J2. All vaccine batches (01, 02, 03) showed steady weight increase (489.8–506.75 g at 20 dpi), consistent

with PBS controls (499.75 g, Table 4). Additionally, serial passaging of Re-H9-DIVA-J2 in embryonated eggs resulted in no embryo lethality,

Table 3
N2-iELISA differentiation of DIVA using NA (N2) antibodies.

Antisera Group	Mean S/N ± SD	Positive Rate (% (n))
Re-H9-DIVA-J2	1.02 ± 0.11	0 (0/10)
WJ57 strain	4.83 ± 0.51	100 (10/10)
GD/J2/2016 strain	5.14 ± 0.63	100 (10/10)
HN03 strain	5.11 ± 0.70	100 (10/10)
H9N2-infected	4.92 ± 0.45	100 (10/10)
PBS	0.89 ± 0.07	0 (0/10)

Note: Data presented as mean ± SD (n = 10). S/N ratio = sample OD / negative control OD. Interpretation: S/N > 2.1 (positive), 1.5–2.1 (suspicious), < 1.5 (negative).

Table 4
Body weight changes in vaccinated groups (Days 0–20).

Vaccine Group	Body weight change (g)				
	0d	5d	10d	15d	20d
01	212.80 ± 8.06	235.65 ± 9.01	302.53 ± 12.36	362.56 ± 22.57	489.8 ± 32.87
02	206.25 ± 6.06	229.70 ± 6.35	312.05 ± 22.04	378.50 ± 31.02	506.75 ± 45.87
03	207.50 ± 6.83	238.65 ± 7.53	309.70 ± 21.12	376.57 ± 25.34	480.50 ± 39.47
PBS	208.40 ± 5.28	234.3 ± 70.36	310.75 ± 16.48	378.5 ± 23.16	499.75 ± 34.10

further underscoring its low pathogenicity and suitability for industrial-scale production.

4. Discussion

The global persistence of H9N2 avian influenza highlights the critical need for vaccines that effectively balance disease control with accurate surveillance—a challenge that conventional inactivated vaccines have failed to address (Carnaccini and Perez, 2020; Dong et al., 2022). Our development of Re-H9-DIVA-J2, featuring a B/Yamagata-lineage NA ectodomain substitution, offers a novel solution to this persistent problem. This approach builds upon existing DIVA strategies while addressing their key limitations. Unlike NS1-truncated vaccines that often compromise immunogenicity due to impaired viral replication (Nogales et al., 2022), or HA2 chimeric designs that risk disrupting critical HA functionality (Kim et al., 2017; Sun et al., 2021), our B-NA substitution strategy leverages the profound antigenic divergence between IAVs and IBVs to ensure clear serological differentiation without sacrificing vaccine efficacy. The retained HA-NA functional balance in Re-H9-DIVA-J2, demonstrated by its high growth titers and genetic stability, overcomes a major hurdle that has plagued previous heterologous NA designs.

The practical advantages of this approach become particularly evident when considering large-scale monitoring applications. While NS1-based assays require specialized reagents, our strategy seamlessly integrates with existing N2-iELISAplatforms—a significant advantage

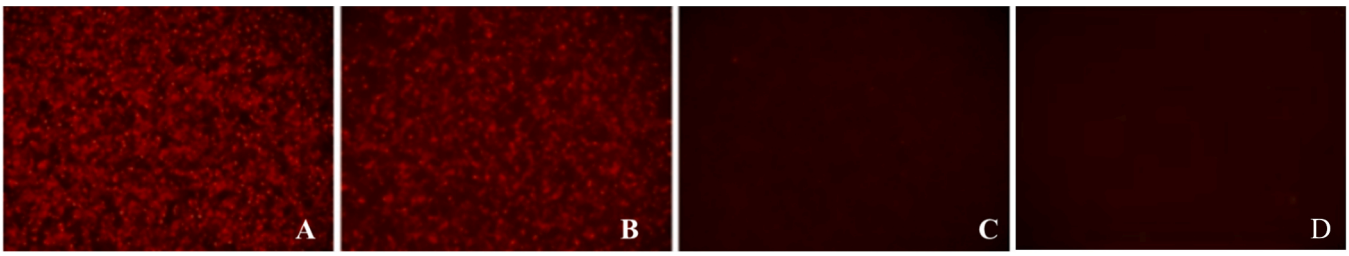

Fig. 5. Reactivity analysis of H9 subtype influenza virus NA (N2) protein expressed in 293 T cells using chicken antisera. (A) H9N2 GD/J2/2016 strain antiserum (positive control); (B) Serum from H9N2-infected chickens; (C) Re-H9-DIVA-J2 antiserum; (D) PBS control (negative control).

for resource-limited regions where H9N2 is endemic. Crucially, the N2-iELISA retained its discriminatory power even after challenge. The seroconversion observed in vaccinated chickens following virus exposure (Table S3) confirms that this DIVA strategy can effectively differentiate between merely vaccinated flocks and those in which vaccination is accompanied by silent field virus circulation. This attribute is indispensable for enabling accurate surveillance and timely intervention in endemic regions where vaccination is practiced. Overall, this compatibility with standard diagnostic infrastructure makes the transition to DIVA-compatible surveillance both economically and technically feasible.

However, certain considerations merit further investigation. Although B-NA antibodies show no cross-reactivity with A-type NA, their potential impact on long-term immune focus remains to be fully understood. Furthermore, the strategy's applicability must be considered in the context of viral diversity. While H9N2 is the dominant subtype, H9 viruses can occasionally reassort with other NA subtypes (e.g., H9N1, H9N3). Our N2-specific companion diagnostic would not serologically identify infections caused by such non-N2 H9 viruses, indicating that the current DIVA system is optimally suited for regions where H9N2 is prevalent. For broader application, future strategies could explore multiplex NA serology. Separately, while the genetic distance between IAVs and IBVs makes NA recombination events unlikely, ongoing monitoring will be essential to assess this theoretical risk in field conditions.

Looking beyond immediate applications, our findings challenge conventional assumptions about the compatibility of A- and B-type influenza proteins. The successful incorporation of B-type NA while maintaining viral fitness opens new possibilities for vaccine design, potentially extending to other problematic subtypes like H5 or H7. The N2-iELISA platform used in this study represents more than just a diagnostic tool—it offers a practical pathway for implementing DIVA strategies at national levels, combining high-throughput capacity with cost-effectiveness that matches the needs of large-scale eradication programs.

As we move forward, the translation of these findings from laboratory to field settings will be crucial. Future work should focus on validating the assay's performance with diverse field sera and educating stakeholders about the benefits of DIVA-compatible approaches. While challenges remain, particularly in ensuring long-term protection and monitoring ecological impacts, this study provides a robust foundation for next-generation avian influenza vaccines. By addressing both immunological and surveillance needs simultaneously, our approach represents a significant step toward the dual goals of protecting poultry health and reducing zoonotic risks—a combination that could transform how we manage avian influenza in endemic regions worldwide.

CRediT authorship contribution statement

Jiasheng Song: Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Huawei Sun:** Writing – review & editing, Methodology. **Fei Yu:** Writing – review & editing, Methodology. **Jinping Fu:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis. **Minhua Sun:** Writing – review & editing, Supervision, Investigation, Formal analysis. **Jingfeng Zhang:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis. **Qunxing Pan:** Writing – review & editing, Visualization, Methodology. **Zhiyu Fan:** Writing – review & editing, Methodology, Investigation, Funding acquisition.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2026.110917.

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