



# Immunogenicity and protective efficacy of an inactivated bivalent vaccine containing two recombinant H1N1 and H3N2 swine influenza virus strains

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## Abstract

The wild-type H1N1 and H3N2 swine influenza virus (SIV) strains are unsuitable for vaccine production because of high lethality in chicken embryos and low reproductive titers. This study developed recombinant H1N1-Re1 and H3N2-Re1 strains via HA and NA genes from the wild-type H1N1 SW/GX/755/17 and H3N2 SW/GX/1659/17 strains combined with six internal genes from the H1N1 A/PR/8/34 strain. The recombinant viruses demonstrated typical cytopathic effects in MDCK cells, and the presence of viral particles was confirmed via electron microscopy. Growth curve analysis revealed titers of  $10^{8.31}$  and  $10^{8.17}$  EID<sub>50</sub> per 100  $\mu$ L for H1N1-Re1 and H3N2-Re1, respectively, within 72–96 h postinoculation. Virus stocks were used to produce a bivalent inactivated vaccine. After two immunizations, hemagglutination inhibition titers in piglets were significantly greater than those induced by commercial vaccines and were sustained from 5 to 29 weeks postimmunization. Upon challenge with virulent wild-type SIV strains, viral isolation occurred in all pigs in the PBS group (5/5 protection), whereas no virus was detected in the bivalent vaccine group (0/5). In contrast, the commercial vaccine group had a viral isolation rate of 1/5. Pathological examination revealed severe pulmonary lesions in the PBS group, mild changes in the commercial vaccine group (1/5), and normal lung morphology in the bivalent vaccine group. This study demonstrated the successful application of an eight-plasmid reverse genetics system to develop recombinant vaccine strains with enhanced immunogenicity and replication efficiency. The bivalent inactivated vaccine provides prolonged and complete protection against H1N1 and H3N2 SIV strains, offering a robust tool for controlling evolving SIV variants.

**Keywords** Swine influenza virus · H1N1 subtype · H3N2 subtype · Immune duration · Protective efficacy

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## Introduction

Swine influenza (SI) is a highly contagious respiratory disease that affects pigs of all ages and is caused by swine influenza virus (SIV) [1]. Clinical symptoms include fever, depression, anorexia, dyspnea, abdominal breathing, and paroxysmal coughing [2]. Pigs can also be infected by swine, avian and human influenza A virus (IAV), which play crucial roles in the “avian-pig-human” transmission chain [3]. Globally, outbreaks of swine influenza result in significant trade restrictions, reduced productivity, and increased costs for disease control, making it a pressing issue for the swine industry. In addition to causing substantial economic losses, the virus also poses a public health risk by contributing to the emergence of zoonotic and potentially pandemic influenza strains.

SIV, a type of IAV, is a negative-sense single-stranded RNA virus [1]. Its genome contains eight segments encoding at least 11 proteins [1]. The viral surface has two glycoproteins: hemagglutinin (HA) and neuraminidase (NA) [4]. SIVs are classified into subtypes on the basis of HA and NA combinations [4]. SIV HA subtypes include H1, H3, H4, H5, H7, and H9, whereas NA subtypes include N1 and N2 [5]. Previous studies have indicated that H1N1 and H3N2 are the most prevalent SIV subtypes in China, causing severe economic losses in the pig industry [6]. One study reported that 228 SIV strains were isolated from over 36,000 pigs, 139 of which were H1N1 subtypes [7]. A serological survey revealed that H3N2 seropositivity in pigs ranged from 1.1 to 47.9% across various regions in China, indicating a high infection rate [8]. Vaccination remains the most effective strategy for preventing and controlling SI, especially in some developing countries [9, 10]. Currently, inactivated vaccines for H1N1 and H3N2 SIV are commercially available in China.

However, current commercial inactivated vaccines provide limited and strain-specific protection, making them less effective against rapidly evolving SIV strains [11]. This study aimed to address the limitations of current SI vaccines by developing a bivalent inactivated vaccine targeting domestic EA H1N1 and H3N2 SIV infections. Two recombinant SIV strains were constructed via reverse genetics technology, which combines the HA and NA genes from circulating strains with internal genes optimized for growth in chicken embryos. The resulting vaccine elicited strong immune responses in pigs, with anti-HA antibody titers reaching 2048 and protective immunity lasting for up to 29 weeks. These findings demonstrate the effectiveness of the vaccine in preventing H1N1 and H3N2 SIV infections and its potential as a practical tool for SI control. Furthermore, the vaccine design strategy offers a flexible and timely

model for addressing emerging SIV strains, contributing to global efforts in SI prevention and control.

## Materials and methods

### Vaccines and viruses

The SW/GX/755/17 and SW/JL/723/17 strains are H1N1 subtype SIVs, and the SW/GX/1659/17 strains are H3N2 subtype SIVs. The A/PR/8/34 strain belongs to the H1N1 subtype and shows good adaptability for chicken embryo reproduction. The recombinant H1N1-Re1 strains and H3N2-Re1 strains have been previously constructed [12, 13]. All SIV strains were isolated, identified, preserved and provided by the Key Laboratory of Animal Influenza, Ministry of Agriculture and Rural Affairs, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. A commercial bivalent inactivated vaccine for swine influenza (H1N1 LN and H3N2 HLJ SIV strains) was purchased from Huaweite (Jiangsu) Biopharmaceutical Co., Ltd.

### Animals

Thirty healthy, weaned piglets, aged four to six weeks, were provided by Shandong Sinder Technology (Weifang, China) Co., Ltd. Prior to randomization, all animals were screened to ensure they met the following exclusion criteria: (i) a clinically healthy condition upon veterinary inspection, (ii) a uniform body weight range of 5–7 kg, and (iii) seronegativity for influenza (H1N1 and H3N2) and other major swine respiratory pathogens. To minimize confounding variables, all piglets were sourced from the same farm with a known biosecurity status. They underwent a quarantine and observation period of seven days to exclude the possibility of subclinical infections. Throughout the study, piglets were housed under the strict standard operating procedures (SOPs), including regular sanitation and controlled access, thus limiting exposure to external pathogens. Ten piglets were randomly assigned to each of the following groups: (1) bivalent inactivated vaccine (H1N1-Re1 + H3N2-Re1), (2) commercial bivalent vaccine (H1N1 LN + H3N2 HLJ), and (3) PBS control. All experimental procedures were approved by the Committee on Ethical Use of Animals of Shandong Sinder Technology (Weifang, China) Co., Ltd (Approval number: SYXK-2023-0037).

## Identification of the recombinant H1N1-Re1 and H3N2-Re1 SIV strains

The procedures for identifying the recombinant H1N1-Re1 and H3N2-Re1 SIV strains were performed as previously described [14]. Briefly, the recombinant H1N1-Re1 and H3N2-Re1 SIV strains were harvested and used to inoculate 10-day-old SPF chicken embryos through the allantoic cavity. At 72 h postinoculation (hpi), the allantoic fluid was harvested, and the HA titers were detected. Additionally, to identify the antigenic changes of the recombinant viruses, the antigenicity between the wild-type and recombinant strains was tested via a cross-HI test with the corresponding sera [15]. The recombinant SIVs were also inoculated into MDCK cells (ATCC CCL-34), and the cytopathic effects were monitored. Additionally, the supernatants from MDCK cells were analyzed via electron microscopy to detect the presence of intact viral particles.

## Virus growth curve and bivalent inactivated vaccine preparation

To determine the growth curves of the recombinant (H1N1-Re1 and H3N2-Re1 strains) and parental (H1N1 SW/GX/755/17 and H3N2 SW/GX/1659/17 strains) SIV strains, each of the viruses was diluted to 100 50% embryo infectious dose ( $EID_{50}$ ) per 100  $\mu$ L and inoculated separately into 60 ten-day-old SPF chicken embryos. At 12, 24, 36, 48, 60 and 72 hpi, 5 chicken embryos were randomly selected, and the allantoic fluid was collected [16]. The allantoic fluid was subsequently diluted in a 10-fold gradient, and five ten-day-old SPF chicken embryos were inoculated with each gradient. After incubation at 37 °C with 64% humidity for 72 h, the allantoic fluid of the chicken embryos was harvested, and the HA titer was subsequently measured. Finally, the  $EID_{50}$  was calculated via the Reed–Muench method [17]. The growth curves of the recombinant and parental SIVs in chicken embryos were plotted according to the  $EID_{50}$  titer at each time point. On the basis of the growth curve characteristics of the recombinant H1N1-Re1 and H3N2-Re1 strains, allantoic fluid containing the viruses was collected when the  $EID_{50}$  titers were at their peak. The allantoic fluids were then inactivated by adding a final concentration of 0.1% formaldehyde solution and incubating at 37 °C for 24 h. Subsequently, the inactivated recombinant H1N1-Re1 and H3N2-Re1 virus fluids were mixed at a volume ratio of 1:1 to prepare the antigen solution. This antigen solution was then thoroughly mixed with ISA 15 A VG adjuvant at a volume ratio (v/v) of 85:15. After stirring at 800 r/min for 30 min, a bivalent inactivated swine influenza vaccine was prepared, ensuring that the hemagglutinin (HA) titers of

both the recombinant inactivated H1N1-Re1 and H3N2-Re1 viruses were 256 per dose.

## Piglet vaccination and serum sample collection

A total of thirty healthy piglets aged 4 to 6 weeks were randomly divided into 3 groups, with 10 piglets in each group in Fig. 3A. In group 1, the 10 piglets were all injected with the bivalent inactivated vaccines (H1N1-Re1 and H3N2-Re1 SIV strains) via the neck muscle. In group 2, the piglets were all injected with the commercial bivalent inactivated vaccines (H1N1 LN and H3N2 HLJ strains) via the same route. In group 3, all the piglets were nonimmunized control piglets. All the pigs in the three groups were intramuscularly administered twice, with a volume of  $10^8$   $EID_{50}$  for each piglet. The interval between each immunization was 3 weeks. Blood samples were collected prior to immunization and at 1, 2, 3, 4, 5, 9, 13, 17, 21, 25 and 29 weeks postimmunization (wpi) in Fig. 3A.

## Pig challenge and immune duration determination

To evaluate the efficacy of the bivalent inactivated vaccine against wild-type SIV infection and to determine its immune duration, each of the three groups was further divided into two equal subgroups at 29 wpi, resulting in a total of six distinct groups in Fig. 3A. At 29 wpi, one subgroup of 5 pigs in each group were challenged via tracheal inoculation with  $10^{8.0}$   $EID_{50}$  doses of the wild-type H1N1 SW/JL/723/17 SIV strain. The other subgroups were challenged via tracheal inoculation with  $10^{8.0}$   $EID_{50}$  doses of the wild-type H3N2 SW/JL/1659/17 SIV strain. Then, histopathological examinations of the lungs were conducted at 3 days post-challenge (dpc), and nasal swabs and lung tissues were collected for virus isolation. Ultimately, the immune protection rate was assessed on the basis of clinical symptoms, lung pathological alterations and virus isolation results.

Serum sample processing and hemagglutination inhibition (HI) assay.

Our cross-HI assays were conducted in accordance with the WHO Global Influenza Surveillance Network guidelines (Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza) insofar as they apply to swine isolates. We used in-house positive serum controls validated against our circulating strains to ensure consistency and reproducibility. The serum samples were subjected to HI testing to detect the presence of anti-H1N1 and H3N2 SIV HA antibodies [15]. First, the serum samples from the pigs were treated with receptor-destroying enzyme (RDE II, Denka Seiken) following the specific operational procedure outlined below: (A) To 1 volume of serum (50  $\mu$ L), 4 volumes of RDE (200  $\mu$ L) were added, and the samples were

incubated in a water bath at 37 °C for 18 h. (B) Five volumes (250 µL) of 1.5% sodium citrate solution were added, mixed thoroughly, and placed in a water bath at 56 °C for 30 min (to inactivate any residual RDE activity). (C) One volume of 50% red blood cells was added to 10 volumes of RDE-treated serum (50 µL) of red blood cells were added to 500 µL of serum, the mixture was gently mixed by shaking, incubated at 4 °C for 1 h, and the red blood cells were gently resuspended several times during this period. (D) The mixture was centrifuged at  $1000 \times g$  for 10 min, the supernatant layer was carefully aspirated, and the mixture was used for subsequent analysis. The final processed serum was diluted 10-fold. Then, 0.025 mL of PBS (0.01 mol/L, pH 7.2) was added to wells 2–11 of a 96-well microtiter plate, and 0.05 mL of PBS was added to well 12 as a control. Pipette 0.05 mL of the treated serum into well 1. Twofold serial dilutions were performed by transferring 0.025 mL of serum from well 1 into well 2, which continued through well 10. Discard 0.025 mL from well 10. Next, an aliquot of antigen containing four hemagglutination units (4 HA units) was added to wells 1 to 11 and allowed to incubate for 30 min at room temperature. Finally, 0.025 mL of a 1% (v/v) chicken red blood cell suspension was added to each well, which was gently mixed and allowed to stand for another 30 min at room temperature. The control erythrocytes exhibited distinctly button-shaped pores. The results were classified as hemagglutination inhibition. Approximately half of the red blood cells agglutinated and formed a thin film at the bottom of the tube, with a small area. The non-agglutinated red blood cells gathered into small dots at the center of the tube. The highest dilution of the agglutinated virus was the agglutination titer, indicating that it contained one unit of hemagglutination antigen.

## Results

### Characterization of the recombinant H1N1-Re1 and H3N2-Re1 SIV strains

The genes of H1N1-Re1 and H3N2-Re1 were derived from the wild-type H1N1 SW/GX/755/17 and H3N2 SW/GX/1659/17 strains with HA and NA, respectively. The other six genes were sourced from the H1N1 A/PR/8/34 strain. The sequencing results revealed that all the recombinant plasmids containing the different H1N1-Re1 genes were successfully constructed (data not shown). After the inoculation of MDCK cells with allantoic fluid containing the recombinant H1N1-Re1 and H3N2-Re1 SIV strains, the cells were enlarged, exhibited nuclear pyknosis or rupture, and exhibited partial or complete detachment (Fig. 1A). Supernatants from inoculated MDCK cells were collected

and analyzed via electron microscopy. The results revealed intact SIV particles, which were characterized by spherical or elliptical two-layered envelopes with diameters ranging from approximately 80 to 120 nm and some protruding spikes on their surfaces (Fig. 1B). Additionally, the results of the serum cross-HI test revealed that the HI titer of the same serum sample against the SW/GX/755/17, SW/GX/1659/17, H1N1-Re1, and H3N2-Re1 SIV strains was 1280, indicating strong antigenicity for both recombinant strains (Fig. 1C and D).

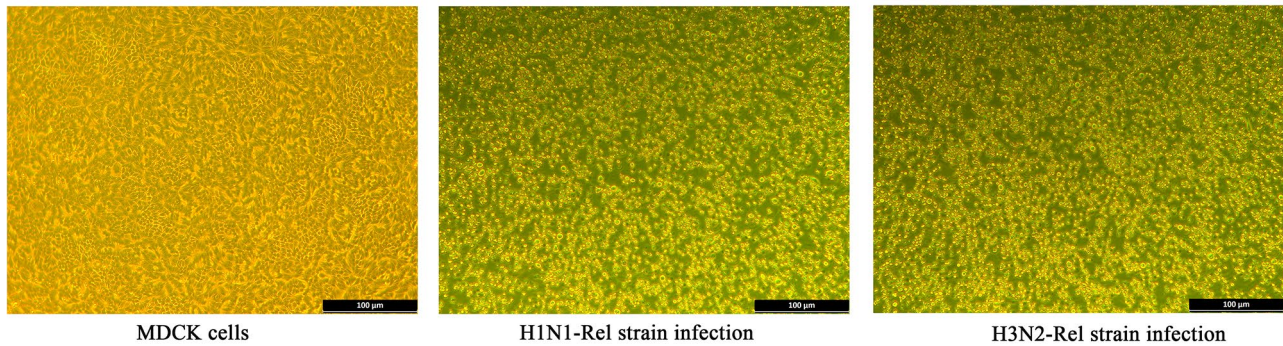
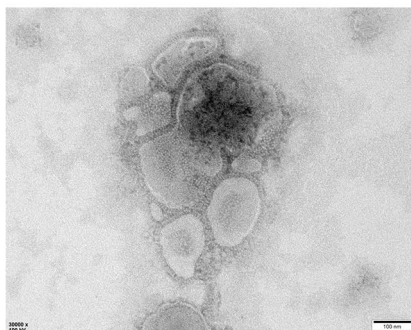
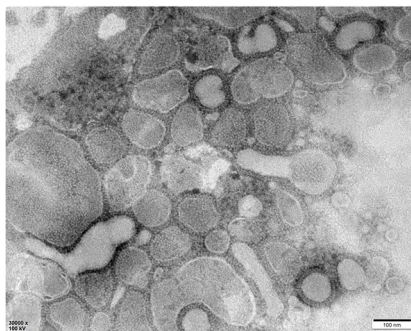
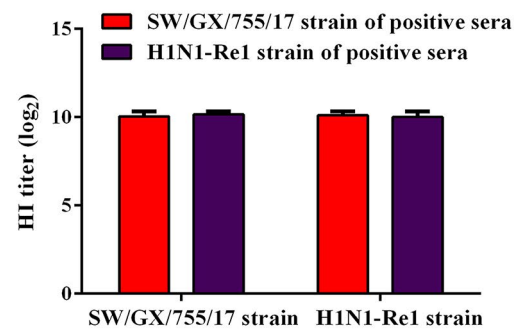
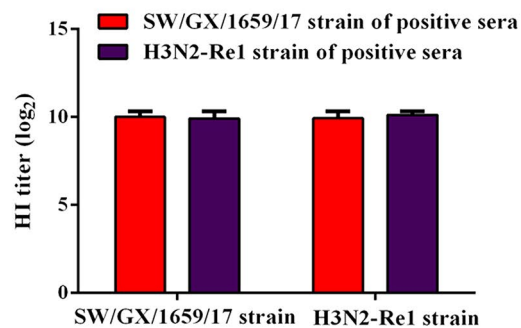
### Determination of the growth curves of the recombinant H1N1-Re1 and H3N2-Re1 SIV strains

Growth curve analysis of the recombinant H1N1-Re1 strain revealed that all the chicken embryos survived for 96 h postinoculation, with a peak titer of  $10^{8.31}$  EID<sub>50</sub> per 100 µL and an HA titer of 512 (Fig. 2A, and 2B). Similarly, in the H3N2-Re1 strain, the titer reached  $10^{8.17}$  EID<sub>50</sub> per 100 µL, with an HA titer of 512 (Fig. 2C, and 2D). In comparison, the wild-type H1N1 SW/GX/755/17 and H3N2 SW/GX/1659/17 strains presented peak titers of  $10^{7.31}$  and  $10^{7.17}$  EID<sub>50</sub> per 100 µL, respectively, with HA titers of 128 for both strains (Fig. 2A, B and C, and 2D). These findings demonstrated that the EID<sub>50</sub> titers of the recombinant strains were approximately 10 times greater than those of their parental wild-type strains, reflecting significantly increased replication ability in chicken embryos. High-growth recombinant H1N1-Re1 and H3N2-Re1 strains were successfully produced, facilitating the development of a bivalent inactivated vaccine targeting the H1N1 and H3N2 subtypes.

### Immune duration of the bivalent inactivated vaccine

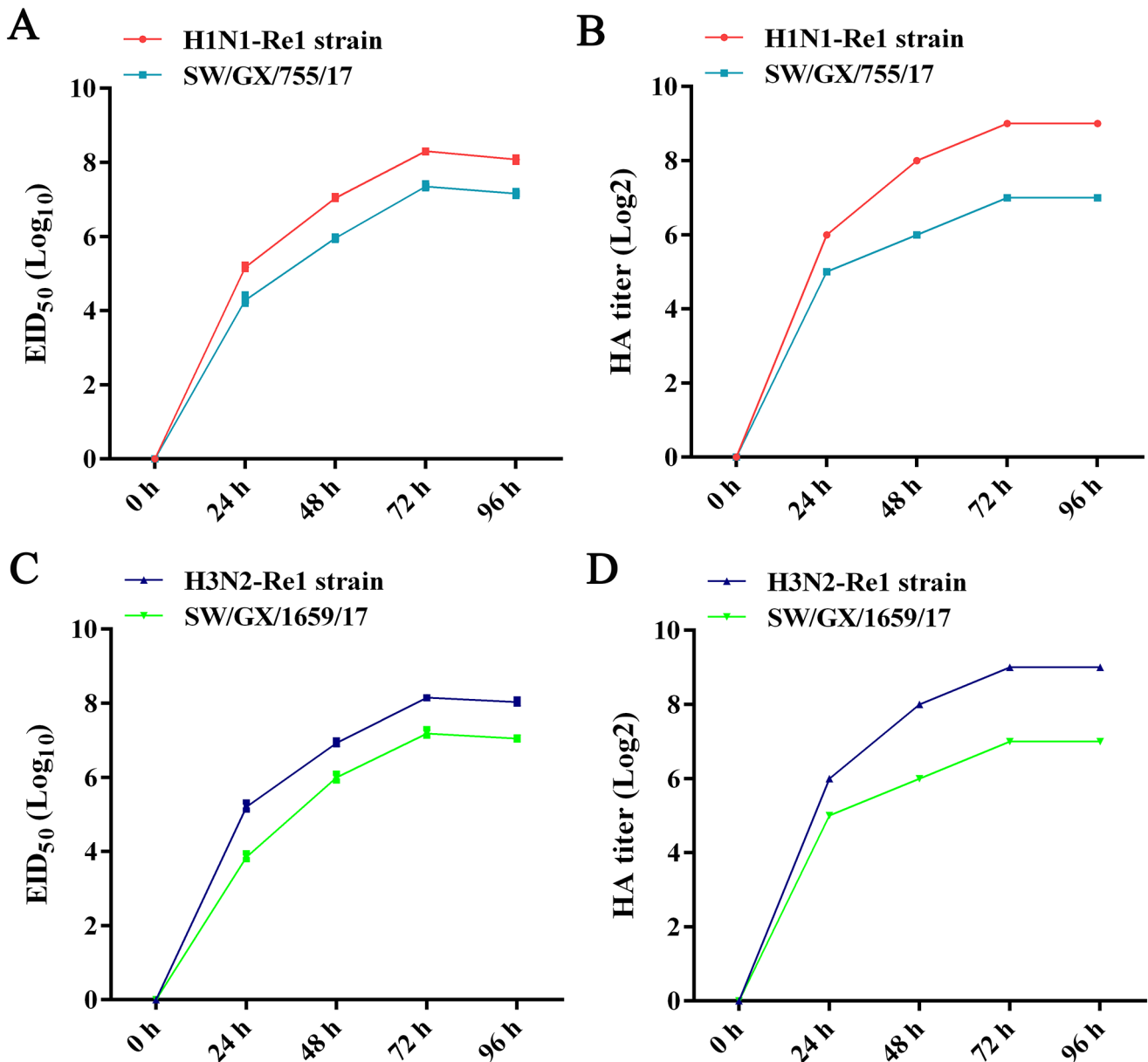
The levels of HI antibodies following immunization with the bivalent inactivated vaccine were assessed. Preimmunization HI antibody levels for both H1N1 and H3N2 SIV were less than 10. At 5 weeks after the first immunization, corresponding to 2 weeks after the second immunization, HI antibody levels peaked (Fig. 3B and C). For the H1N1 SIV, the HI ranged from 1280 to 5120 (mean: 2368), whereas, for H3N2 SIV, the HI ranged from 1280 to 5120 (mean: 2304) (Fig. 3A and B). At 29 wpi, the HI levels were still between 80 and 320 (average 144) for H1N1 SIV and between 80 and 320 (mean: 152) for H3N2 SIV (Fig. 3B and C). For the groups with the commercial vaccines (H1N1 LN+H3N2 HLJ SIV strains), the HI levels of H1N1 SIV ranged from 640 to 2560 (mean: 1056) at 5 wpi and the HI levels of H3N2 SIV ranged from 640 to 2560 (mean: 1088). By 29 wpi, HI levels declined to 40 to 160 for H1N1 SIV (mean: 68) and 40 to 160 for H3N2 SIV (mean: 152) (Fig. 3B and 3C). These



**A****B****C****D**

**Fig. 1** Characterization of Recombinant Swine Influenza Viruses for Bioactivity Assessment. **(A)** Cytopathic effects in MDCK cells infected with the recombinant H1N1-Re1 and H3N2-Re1 swine influenza virus (SIV) strains. **(B)** Transmission electron microscopy images of purified H1N1-Re1 and H3N2-Re1 SIV particles. Viral particles were negatively stained with 1% phosphotungstic acid, as described

in Sect. [Materials and methods](#). Scale bar: 100 nm. **(C)** Serum cross-HI test results for H1N1-Re1. The HI titers of the same serum sample against SW/GX/755/17, SW/GX/1659/17, and H1N1-Re1 are shown. **(D)** Serum cross-HI test results for H3N2-Re1. The HI titers of the same serum sample against SW/GX/755/17, SW/GX/1659/17, and H3N2-Re1 are shown



**Fig. 2** Growth curves of the SW/GX/755/17, SW/GX/1659/17, H1N1-Re1, and H3N2-Re1 SIV strains in 10-day-old SPF chicken embryos. (A) Growth curves of the SW/GX/755/17 and H1N1-Re1 SIV strains. (B) HA titers for the SW/GX/755/17 and H1N1-Re1 SIV strains. (C) Growth curves of the SW/GX/1659/17 and H3N2-Re1 SIV strains.

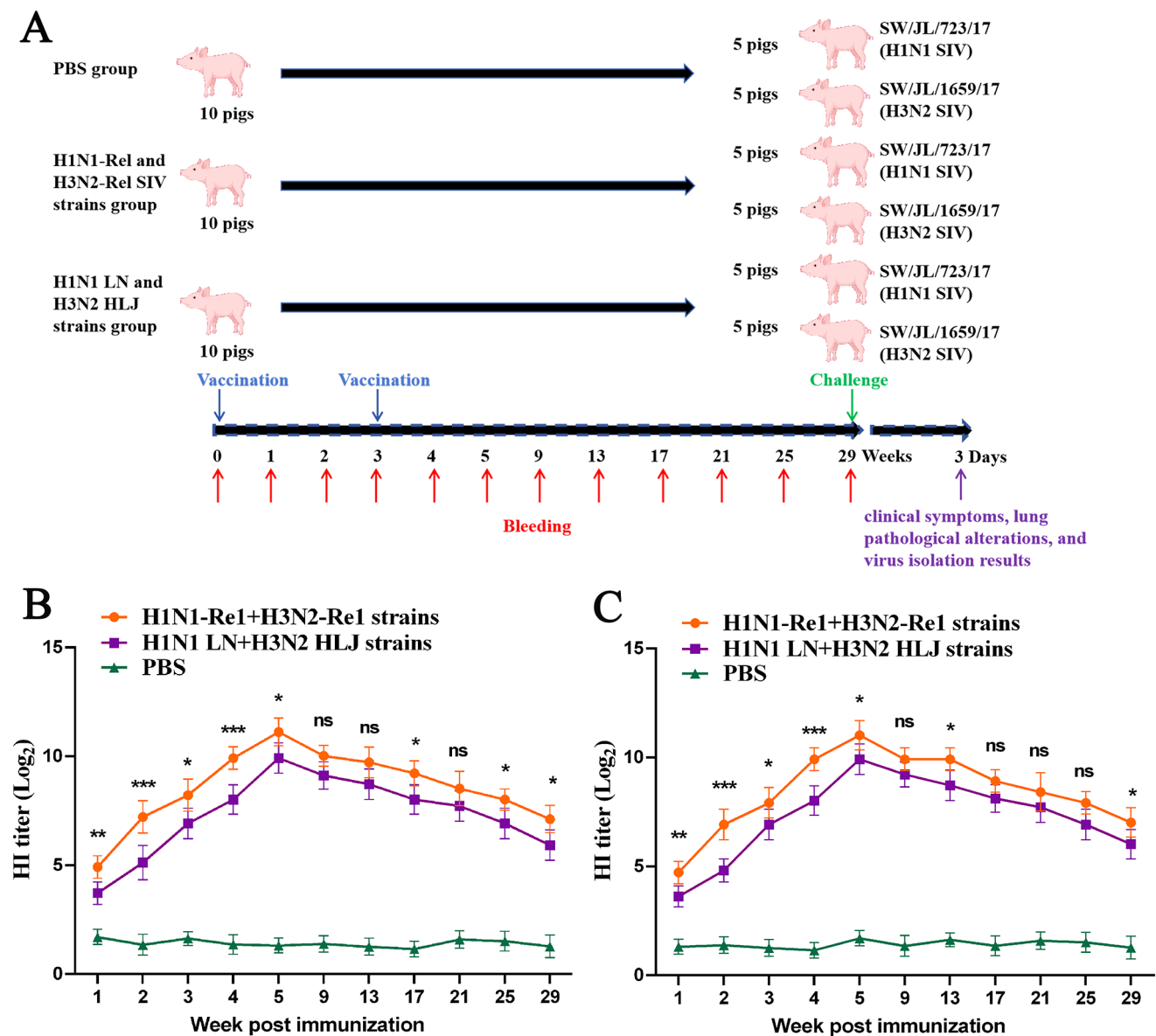
(D) HA titers for the SW/GX/1659/17 and H3N2-Re1 SIV strains. The viral titers of the SW/GX/755/17, SW/GX/1659/17, H1N1-Re1, and H3N2-Re1 SIV strains were measured in 10-day-old SPF chicken embryos. Viral growth was assessed at different time points postinoculation, and titers were determined as EID<sub>50</sub> per 100  $\mu$ L.

results showed that the HI titers of the bivalent inactivated vaccine group were greater than those of the commercial vaccine group.

### Protection of the bivalent inactivated vaccine containing the recombinant H1N1-Re1 and H3N2-Re1 SIV strains

After the pigs were inoculated with the  $10^{8.0}$  EID<sub>50</sub> of the wild-type H1N1 SW/JL/723/17 or H3N2 SW/GX/1659/17

strains, the results revealed that all the pigs in the bivalent inactivated vaccine group were negative for virus isolation, indicating that these pigs obtained complete protection against viral challenge (Fig. 4A). In contrast, four out of five pigs in the commercial vaccine group tested negative for virus isolation, demonstrating an 80% (4/5) protection (Fig. 4A). In the PBS group, all the pigs were positive for virus isolation (Fig. 4A). Furthermore, the pigs in the PBS group exhibited clinical manifestations of lung involvement, including edema and hemorrhage, with pathological



**Fig. 3** The HI reactivity of serum samples following vaccination with the bivalent inactivated vaccine. **(A)** Schematic representation of the experimental setup for evaluating the immunogenicity and duration of the bivalent inactivated vaccine against wild-type SIV infection. At 29 wpi, each of the three experimental groups was further divided into

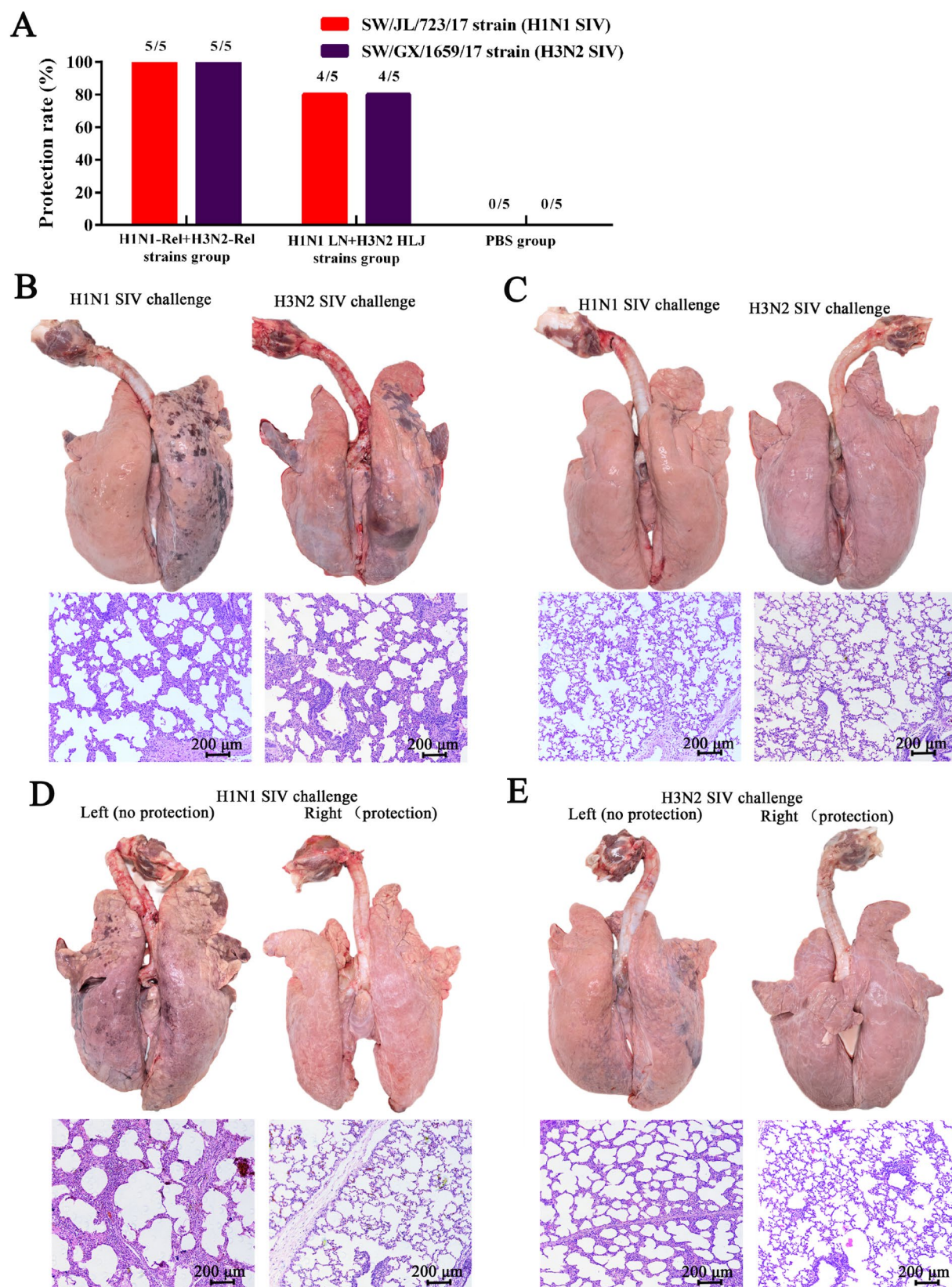
two equal subgroups, resulting in six distinct groups. **(B)** HI reactivity of serum samples against the H1N1 SW/JL/723/17 SIV strain following vaccination. **(C)** HI reactivity of serum samples against the H3N2 SW/JL/1659/17 SIV strain following vaccination

examination revealing thickening of the alveolar walls accompanied by hemorrhage (Fig. 4B). Among the pigs in the commercial vaccine group, one pig exhibited mild pulmonary edema, with histopathological analysis indicating alveolar wall thickening, and the other pigs presented no abnormalities (Fig. 4D and E). In contrast, the pigs in the bivalent inactivated vaccine group presented normal lung morphology (Fig. 4C). These results indicated that the bivalent inactivated vaccine can provide complete protection against viral challenge in piglets and was more effective than the commercial vaccine.

## Discussion

An ideal swine influenza vaccine strain should meet three key criteria [17]: strong antigenic specificity, aligning with epidemic strains [18]; good adaptability to chicken embryos, allowing high-titer virus propagation [19]; and low pathogenicity to pigs, humans, and other livestock [20]. Therefore, once a wild-type SIV strain is isolated, it must be attenuated and adapted to chicken embryos to produce inactivated vaccines on a large scale [20]. Three aspects, including antigenic specificity, biosafety and chick embryo growth







**Fig. 4** Protective efficacy of the bivalent inactivated vaccine against the virulent H1N1 SW/JL/723/17 and H3N2 SW/GX/1659/17 SIV strains. **(A)** Protection rates of the bivalent inactivated vaccine (H1N1-Re1 + H3N2-Re1) compared with those of the commercial vaccine (H1N1 LN + H3N2 HLJ) and the PBS group. The bivalent inactivated vaccine provided 100% protection against both the H1N1 and H3N2 strains, whereas the commercial vaccine group achieved 80% protection. No protection was observed in the PBS group. **(B)** Gross pathological and histopathological changes in the PBS group following the H1N1 or H3N2 SIV challenge. The lungs exhibit severe pulmonary edema, hemorrhage, and thickened alveolar walls. **(C)** Gross and histopathological findings in the bivalent vaccine group (H1N1-Re1 + H3N2-Re1) following the H1N1 or H3N2 SIV challenge. Normal lung morphology was observed, indicating complete protection. **(D)** Gross and histopathological findings in the commercial vaccine group (H1N1 LN + H3N2 HLJ) following challenge with H1N1 SIV. The lungs exhibited partial protection, with evidence of mild edema and thickened alveolar walls (right: protection; left: no protection). **(E)** Gross and histopathological findings in the commercial vaccine group (H1N1 LN + H3N2 HLJ) following challenge with H3N2 SIV. The lungs exhibit partial protection, with mild pathological changes (right: protection; left: no protection)

adaptability, are important in the production of inactivated influenza virus vaccines [21]. The wild-type SIV isolates have ideal antigenic specificity, but they have some negative sides for directly producing the inactivated vaccine, including biosafety and poor growth adaptability in chicken embryos [22]. In recent years, the reverse genetic manipulation of the influenza virus has become an important technique for investigating the functions of viral proteins [23]. For IAV rescue, an eight- or twelve-plasmid reverse genetic operating system is usually used [12]. In the present study, an eight-plasmid reverse genetic operating system was chosen to rescue SIV. The eight plasmids all contained promoters and terminators recognizable by RNA polymerase I, and the cDNAs for each segmented genome of SIV were placed between the promoters and terminators. A total of 10 proteins of SIV can be expressed by the 8 plasmids and enhance the rescue efficiency of SIV. The six internal genes were from the H1N1 A/PR/8/34 strain, which grows well in MDCK cells and chicken embryos. Our results suggested that the six internal genes of the A/PR/8/34 strain are versatile and can be combined with the HA and NA genes of other SIV strains to generate recombinant viruses. Importantly, the recombinant virus still retained the growth characteristics of the A/PR/8/34 strain in MDCK cells and chicken embryos. Moreover, the recombinant virus containing the HA and NA genes from the prevalent SIV strains acquired the antigenicity of epidemic strains and provided convenience for vaccine development. Our results also imply that when a new epidemic strain emerges in the field, a vaccine against the epidemic strain can be developed rapidly via the eight-plasmid system. Recent surveillance data indicate that H1N1 SW/GX/755/17 and H3N2 SW/GX/1659/17 predominate in several swine-producing regions of southern China, showing antigenic divergence from earlier vaccine strains

(unpublished data). Thus, these isolates were prioritized for reverse-genetics-based vaccine development.

By utilizing specific positive sera for anti-H1N1 and H3N2 antibodies, cross-HI tests revealed that both recombinant SIV strains presented titers of 1280, indicating robust immunogenicity [24, 25]. Growth curve analysis revealed that the titers of H1N1-Re1 and H3N2-Re1 peak at  $10^{8.31}$  EID<sub>50</sub> and  $10^{8.17}$  EID<sub>50</sub> per 100  $\mu$ L, respectively, within 72 to 96 hpi. Additionally, the recombinant SIV strains also efficiently replicated in chicken embryos, with the viral titers in allantoic fluid increasing tenfold compared with those of the wild-type SW/GX/755/17 and SW/GX/1659/17 strains. Consequently, inactivated vaccines can be directly prepared from allantoic fluid containing recombinant viruses without necessitating further virus concentration. The production cost has been significantly reduced, particularly for animal vaccines, demonstrating promising market application potential.

In comparing the protective efficacy of our bivalent inactivated vaccine (100% protection) with that of the commercial vaccine (80% protection), it is important to note that both formulations produced relatively high protection rates. Nonetheless, this additional 20% improvement in the bivalent vaccine group may have considerable epidemiological significance under field conditions, where even modest gains in protection can substantially reduce viral spread and economic losses. One likely explanation for the observed increase in efficacy is the closer antigenic match between our newly generated reassortant strains (H1N1-Re1 and H3N2-Re1) and the circulating swine influenza viruses, since these strains were selected based on recent epidemiological data. Another contributing factor could be the robust immune response induced by combining the HA and NA gene segments of prevalent SIVs with the well-adapted A/PR/8/34 internal genes, which not only improves replication in chicken embryos but may also promote a stronger immunogenic profile. Overall, these findings highlight the practical benefits of continually updating vaccine strains to keep pace with evolving SIV variants and reinforce the viability of our bivalent inactivated vaccine for broad, long-term control of swine influenza.

Recent findings have highlighted important host factors involved in influenza pathogenesis and host defense. For instance, TRIM29 deficiency has been shown to protect against lethal respiratory infections caused by influenza virus [26], and loss of TRIM29 similarly confers protection against viral myocarditis via the regulation of PERK-mediated endoplasmic reticulum (ER) stress immune responses [27]. Additionally, PARP9 has emerged as a noncanonical sensor for RNA viruses, contributing to antiviral immunity [28]. These insights underscore the complex cellular and molecular mechanisms that can influence outcomes

in influenza infection, including secondary complications such as viral myocarditis. While our current study did not specifically measure TRIM29 or PARP9 expression levels, our findings—particularly the robust immune protection observed in piglets—raise intriguing questions about whether this inactivated bivalent vaccine might indirectly modulate these pathways. Future research could investigate the extent to which vaccine-induced immune responses affect TRIM29 and PARP9 activity in swine, thereby offering a more comprehensive view of the molecular mechanisms through which the vaccine confers protection. Such investigations may ultimately pave the way for improved vaccine designs that harness or enhance specific host-defense pathways against emerging influenza variants.

SIV infection can cause weight loss in pigs, which are easily coinfecting with other pathogens, resulting in a lower feed/meat ratio in pigs, decreased reproductive performance and increased mortality. SIV of its counterpart is characterized by coinfection between humans and animals, and there is a risk of virus mutation and cross-species transmission. Hence, the swine influenza epidemic also poses a potential threat to public health. Vaccination is an effective means to prevent SIV infection. SIV vaccine has been widely applied not only in China but also around the world. However, owing to the diversity of viruses, cross-protection among different subtypes is weak. Therefore, farms should carry out immunization in accordance with the epidemic situation of SIV. The current vaccines can shorten the duration of infection and reduce the viral load in lung tissue but do not offer 100% protection 29 weeks after immunization. In this study, immunization studies involving piglets revealed significantly greater dynamic changes in antibody HI titers over a period extending from week five through week twenty-nine than did commercial control vaccines currently available on the market. Notably, challenge protection results indicated that after primary immunization lasting up to twenty-nine weeks, the bivalent vaccine conferred complete protection against the prevalent virulent strains SW/JL/723/17 (H1N1 SIV) and SW/JL/1659/17 (H3N2 SIV), achieving a remarkable success rate that contrasted with the 80% efficacy observed among existing commercial options. Thus, the bivalent inactivated vaccine showed a longer duration of immune protection and better protection than the currently available commercial vaccines.

Although this study has obtained preliminary results, certain deficiencies persist. Research on the immune protection mechanism of vaccines is insufficient, and assessments of the stability, safety, and efficacy of vaccines in diverse environments are not comprehensive enough. To broaden the evaluation scope of the stability and safety of the vaccine in different environments, stability tests under various conditions, such as temperature, humidity, and light, and safety

evaluations of the vaccine in pigs of different breeds, ages, and health statuses are needed. The vaccination schedule should be further optimized to enhance the immune effect and duration of the vaccine. Different vaccination doses, intervals, and routes were explored to identify the optimal immunization regimen. While our vaccine specifically targets dominant Chinese SIV strains, the H1N1 and H3N2 subtypes are widespread globally. Adapting the reverse genetics strategy to incorporate locally circulating HA/NA genes could make the vaccine relevant to other regions. Future cross-reactivity or sequence homology studies would help define its broader applicability.

In conclusion, these findings underscore the effectiveness of employing an eight-plasmid reverse genetics system, leading to the successful construction of recombinant vaccine candidates H1N1-Re1 and H3N2-Re1 strains that are antigenically identical to epidemic SIV strains. The excellent immune protection and persistence of the bivalent inactivated vaccine against the epidemic SIV strains were evaluated in animal experiments. An eight-plasmid reverse genetics system can serve as a reference for other prevalent IAVs and offers support for the development of vaccines against SIV strains. The bivalent inactivated vaccine developed concurrently has promising market application prospects in the prevention and control of SI.

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**Author contributions** Heng Zhang: Conceptualization, Methodology, Validation, Data curation, Writing—original draft, Funding acquisition. Xu Chen: Data curation, Formal analysis, Validation. Dongying Liu, Xinyu Liu, Yifan Ge and Yani Sun: Investigation, Data curation. Xiaoyue Zhang, Guangen Hao, Zhaoyang Li, Qingqing Song and Lei Wang: Formal analysis, Validation. Zhao Wang, Huanliang Yang and Qing Pan: Methodology, Writing—review & editing, Qin Zhao: Conceptualization, Methodology, Writing—review & editing.

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**Data availability** The data that support the study results are available upon reasonable request. Corresponding authors should be contacted if data from this study are required.

## Declarations

**Ethics approval and consent to participate** Animal experiments were performed based on the Guidance for Experimental Animal Welfare and Ethical Treatment by the Ministry of Science and Technology of China. The protocols of animal experimental procedures were carried out following the guidelines of the Shandong Sinder Technology (Weifang, China) Co., Ltd Institutional Committee for the Care and Use of Laboratory Animals and were approved by the Committee on Ethical Use of Animals of Shandong Sinder Technology (Weifang, China) Co., Ltd (Approval number: SYXK-2023-0037).

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**Competing Interests** The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## References

- Paules CI, Sullivan SG, Subbarao K, Fauci AS (2018) Chasing seasonal influenza - The need for a universal influenza vaccine. *N Engl J Med* 378(1):7–9. <https://doi.org/10.1056/NEJMp1714916>
- Pizzolla A, Nguyen TH, Sant S, Jaffar J, Loudovaris T, Manering SI, Thomas PG, Westall GP, Kedzierska K, Wakim LM (2018) Influenza-specific lung-resident memory T cells are proliferative and polyfunctional and maintain diverse TCR profiles. *J Clin Invest* 128(2):721–733. <https://doi.org/10.1172/JCI96957>
- Zens KD, Chen JK, Farber DL (2016) Vaccine-generated lung tissue-resident memory T cells provide heterosubtypic protection to influenza infection. *JCI Insight* 1(10):e85832. <https://doi.org/10.1172/jci.insight.85832>
- Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, Bean T, Barclay W, Deeks JJ, Lalvani A (2013) Cellular immune correlates of protection against symptomatic pandemic influenza. *Nat Med* 19(10):1305–1312. <https://doi.org/10.1038/nm.3350>
- Osbyer K, Berg M, Sokerya S, Chheng K, San S, Davun H, Magnusson U, Olsen B, Zohari S (2017) Influenza A virus in backyard pigs and poultry in rural Cambodia. *Transbound Emerg Dis* 64(5):1557–1568. <https://doi.org/10.1111/tbed.12547>
- Weinfurter JT, Brunner K, Capuano SV 3rd, Li C, Broman KW, Kawaoka Y, Friedrich TC (2011) Cross-reactive T cells are involved in rapid clearance of 2009 pandemic H1N1 influenza virus in nonhuman primates. *PLoS Pathog* 7(11):e1002381. <https://doi.org/10.1371/journal.ppat.1002381>
- Koutsakos M, Illing PT, Nguyen THO, Mifsud NA, Crawford JC, Rizzetto S, Eltahla AA, Clemens EB, Sant S, Chua BY, Wong CY, Allen EK, Teng D, Dash P, Boyd DF, Grzelak L, Zeng W, Hurt AC, Barr I, Rockman S, Kedzierska K (2019) Human CD8+ T cell cross-reactivity across influenza A, B and C viruses. *Nat Immunol* 20(5):613–625. <https://doi.org/10.1038/s41590-019-0320-6>
- Si L, Xu H, Zhou X, Zhang Z, Tian Z, Wang Y, Wu Y, Zhang B, Niu Z, Zhang C, Fu G, Xiao S, Xia Q, Zhang L, Zhou D (2016) Generation of influenza A viruses as live but replication-incompetent virus vaccines. *Sci (New York N Y)* 354(6316):1170–1173. <https://doi.org/10.1126/science.aah5869>
- Wang L, Liu SY, Chen HW, Xu J, Chapon M, Zhang T, Zhou F, Wang YE, Quanquin N, Wang G, Tian X, He Z, Liu L, Yu W, Sanchez DJ, Liang Y, Jiang T, Modlin R, Bloom BR, Li Q, Cheng G (2017) Generation of a live attenuated influenza vaccine that elicits broad protection in mice and ferrets. *Cell Host Microbe* 21(3):334–343. <https://doi.org/10.1016/j.chom.2017.02.007>
- Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A (2009) Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 459(7250):1122–1125. <https://doi.org/10.1038/nature08182>
- Zhao X, Shen M, Cui L, Liu C, Yu J, Wang G, Erdeljan M, Wang K, Chen S, Wang Z (2024) Evolutionary analysis of hemagglutinin and neuraminidase gene variation in H1N1 swine influenza virus from vaccine intervention in China. *Sci Rep* 14(1):28792. <https://doi.org/10.1038/s41598-024-80457-4>
- Song Zuchen M, Fei Z, Dandan W, Feifei L, Yaru C, Chuanling YQ, Hualan C, Yang Huanliang (2021) Development and identification of high-replicated swine influenza virus GX1659/PR8 reassortant virus. *Chin J Prev Veterinary Med* 43(10):1103–1107 (in Chinese)
- Wang Z, Yang H, Chen Y, Tao S, Liu L, Kong H, Ma S, Meng F, Suzuki Y, Qiao C, Chen H (2017) A Single-Amino-Acid substitution at position 225 in hemagglutinin alters the transmissibility of Eurasian Avian-Like H1N1 swine influenza virus in Guinea pigs. *J Virol* 91(21):e00800–e00817. <https://doi.org/10.1128/JVI.00800-17>
- Sui J, Yang D, Qiao C, Xu H, Xu B, Wu Y, Yang H, Chen Y, Chen H (2016) Protective efficacy of an inactivated Eurasian avian-like H1N1 swine influenza vaccine against homologous H1N1 and heterologous H1N1 and H1N2 viruses in mice. *Vaccine* 34(33):3757–3763. <https://doi.org/10.1016/j.vaccine.2016.06.009>
- Ruan BY, Yao Y, Wang SY, Gong XQ, Liu XM, Wang Q, Yu LX, Zhu SQ, Wang J, Shan TL, Zhou YJ, Tong W, Zheng H, Li GX, Gao F, Kong N, Yu H, Tong GZ (2020) Protective efficacy of a bivalent inactivated reassortant H1N1 influenza virus vaccine against European avian-like and classical swine influenza H1N1 viruses in mice. *Vet Microbiol* 246:108724. <https://doi.org/10.1016/j.vetmic.2020.108724>
- PIZZI M (1950) Sampling variation of the 50% end-point, determined by the Reed-Muench (Behrens) method. *Hum Biol* 22(3):151–190
- Ablasser A, Goldeck M, Cavlar T, Deimling T, Witte G, Röhl I, Hopfner KP, Ludwig J, Hornung V (2013) cGAS produces a 2'-5'-linked Cyclic dinucleotide second messenger that activates STING. *Nature* 498(7454):380–384. <https://doi.org/10.1038/nature12306>
- Wu J, Sun L, Chen X, Du F, Shi H, Chen C, Chen ZJ (2013) Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Sci (New York N Y)* 339(6121):826–830. <https://doi.org/10.1126/science.1229963>
- Wang J, Li P, Wu MX (2016) Natural STING agonist as an ideal adjuvant for cutaneous vaccination. *J Invest Dermatol* 136(11):2183–2191. <https://doi.org/10.1016/j.jid.2016.05.105>
- Corrales L, Glickman LH, McWhirter SM, Kanne DB, Sivick KE, Katibah GE, Woo SR, Lemmens E, Banda T, Leong JJ, Metchette K, Dubensky TW, Jr, Gajewski TF (2015) Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity. *Cell Rep* 11(7):1018–1030. <https://doi.org/10.1016/j.celrep.2015.04.031>
- Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K (2005) Molecular basis of replication of Duck H5N1 influenza viruses in a mammalian mouse model. *J Virol* 79(18):12058–12064. <https://doi.org/10.1128/JVI.79.18.12058-12064.2005>

22. Li IW, Chan KH, To KW, Wong SS, Ho PL, Lau SK, Woo PC, Tsoi HW, Chan JF, Cheng VC, Zheng BJ, Chen H, Yuen KY (2009) Differential susceptibility of different cell lines to swine-origin influenza A H1N1, seasonal human influenza A H1N1, and avian influenza A H5N1 viruses. *J Clin Virology: Official Publication Pan Am Soc Clin Virol* 46(4):325–330. <https://doi.org/10.1016/j.jcv.2009.09.013>
23. Zhou B, Donnelly ME, Scholes DT, George S, Hatta K, Kawakita M, Y., Wentworth DE (2009) Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and swine origin human influenza A viruses. *J Virol* 83(19):10309–10313. <https://doi.org/10.1128/JVI.01109-09>
24. Wang J, Li B, Wu MX (2015) Effective and lesion-free cutaneous influenza vaccination. *Proc Natl Acad Sci USA* 112(16):5005–5010. <https://doi.org/10.1073/pnas.1500408112>
25. Wang J, Shah D, Chen X, Anderson RR, Wu MX (2014) A micro-sterile inflammation array as an adjuvant for influenza vaccines. *Nat Commun* 5:4447. <https://doi.org/10.1038/ncomms5447>
26. Xing J, Weng L, Yuan B, Wang Z, Jia L, Jin R, Lu H, Li XC, Liu YJ, Zhang Z (2016) Identification of a role for TRIM29 in the control of innate immunity in the respiratory tract. *Nat Immunol* 17(12):1373–1380. <https://doi.org/10.1038/ni.3580>
27. Wang J, Lu W, Zhang J, Du Y, Fang M, Zhang A, Sungcad G, Chon S, Xing J (2024) Loss of TRIM29 mitigates viral myocarditis by attenuating PERK-driven ER stress response in male mice. *Nat Commun* 15(1):3481. <https://doi.org/10.1038/s41467-024-44745-x>
28. Xing J, Zhang A, Du Y, Fang M, Minze LJ, Liu YJ, Li XC, Zhang Z (2021) Identification of poly(ADP-ribose) polymerase 9 (PARP9) as a noncanonical sensor for RNA virus in dendritic cells. *Nat Commun* 12(1):2681. <https://doi.org/10.1038/s41467-021-23003-4>

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