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RESEARCH ARTICLE

Evaluation of the Protective Efficacy of Swine Influenza Bivalent Inactivated Vaccine Against Epidemic Strains of H1N1 and H3N2 Swine Influenza Viruses

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ABSTRACT

The protective effect of swine influenza bivalent inactivated vaccine (H1N1 AH strain+H3N2 JS strain) on the epidemic strain of swine influenza virus was evaluated in the present study. Healthy susceptible piglets aged 4 to 5 weeks were immunized twice (with an interval of 2 weeks). Two weeks after the booster immunization, blood samples were collected and tested for HI antibody levels using vaccine strains SW/AH/17, SW/JS/17, as well as epidemic strains SW/GD/21 and SW/SD/22. Then, two weeks after the booster immunization, epidemic strains SW/GD/21 and SW/SD/22 were used for challenge. After challenge, clinical symptoms were observed daily, body temperatures were measured, and nasal swabs were collected for 3-5 days to detect detoxification. On the 5th day, all experimental animals were autopsied to observe the degree of lung injury. The results showed that on the 14th day after the booster immunization, all piglets in the immunized group produced high levels of antibodies against H1 and H3 subtypes, but the antibody levels detected against the epidemic strains were 1-2 titer lower than those of the vaccine strains. After challenge with the epidemic strains, except for one piglet in the SW/GD/21 challenge group with a body temperature exceeding 40.2℃ and detoxification detected, no significant respiratory symptoms were observed in the remaining immunized piglets, and no detoxification was detected. No typical pathological damage was observed in the lungs. Compared with the unimmunized control group, the immunized piglets after challenge with the epidemic strains showed reduced respiratory symptoms caused by swine influenza virus infection, blocked continuous detoxification to the outside world, and significantly reduced pathological damage in the lungs. The study results showed that the swine influenza bivalent inactivated vaccine (H1N1 AH strain + H3N2 JS strain) can provide good protection against both H1N1 and H3N2 epidemic strains.

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INTRODUCTION

Swine influenza (SI) is an acute, contact respiratory disease of pigs caused by swine influenza virus (SIV), which is clinically characterized by fever, runny nose, cough, and loss of appetite. SIV infection has the characteristics of high morbidity and low mortality, but SIV infection can cause reduced feed utilization and growth retardation. At the same time, SIV may also be co-infected with other pathogens, resulting in severe symptoms and death (Dobrescu *et al.,* 2014; Kumar e*t al.,* 2011; Schmidt *et al.,* 2016). The occurrence and prevalence of swine influenza have caused significant economic losses to the pig industry.

Like avian influenza virus, SIVs have different subtypes and strains. At present, H1N1, H1N2 and H3N2 are the three most common subtypes of SIVs circulating in pigs (Cai *et al.,* 2022; Chauhan and Gordon, 2020; Cui *et al.,* 2024). Based on the origin of the viral gene fragments, SIVs can be divided into multiple lineages. In recent years, the classical swine H1N1 (CS H1N1), Eurasian avian-like H1N1 (EA H1N1), and human-like H3N2 strains have been prevalent in Chinese pig herds (Liang *et al.,* 2014; Qiao *et al.,* 2014; Sui *et al.,* 2016; Chen *et al.,* 2024). Vaccination is the most effective

and economical means to prevent and control animal influenza infection. At present, there are many inactivated swine influenza vaccines on the market, but the variation characteristics of influenza virus often cause the mismatch between epidemic strains and vaccine strains, which will reduce the protective effect of the vaccine (Wen *et al.,* 2014; Tenforde *et al.,* 2020; Ryt-Hansen *et al.,* 2021). Therefore, it is necessary to screen and replace the vaccine strains with good protective effect against epidemic strains according to the epidemic characteristics of influenza virus.

The preliminary research results of this laboratory showed that the bivalent inactivated vaccine against swine influenza prepared with these two strains had good protective effect against homologous virus challenge after vaccination in piglets. However, further research is needed to determine whether the vaccine can provide complete protection against the infection of swine influenza virus epidemic strains isolated in recent years. Therefore, in this study, after vaccination of piglets with the swine influenza bivalent inactivated vaccine (H1N1 AH strain+H3N2 JS strain), the H1N1 subtype and H3N2 subtype swine influenza virus epidemic strains were used for challenge to evaluate the antibody production and protective effect against epidemic strains after vaccination.

MATERIALS AND METHODS

Viruses: Four SIVs were used in this study: A/swine/Anhui/CZ13/2017(H1N1), SW/AH/17; A/swine/Jiangsu/DT33/2017(H3N2), SW/JS/17; A/Swine/GuangDong/GZ03/2021(H1N1), SW/GD/21 and A/Swine/ShanDong/LY07/2022(H3N2), SW/SD/22. These viruses were previously isolated from pigs during surveillance activities conducted in China between 2017 and 2022 for swine influenza. SW/AH/17 and SW/JS/17 were used as vaccine viruses, SW/GD/21 and SW/SD/22 were used as challenge viruses in this study. These viruses were propagated in Madin-Darby canine kidney (MDCK) cell and titrated to determine the 50% tissue culture infective dose ($TCID_{50}$) by the method of Reed and Muench.

Adjuvant: Montanide TM GEL02 PR, Seppic, Paris, France.

Laboratory facilities: All experiments involving live H1N1 and H3N2 viruses were conducted within enhanced animal biosafety level 2 plus (ABSL2+) facilities at Sinovet (Jiangsu) Biopharm. Co., Ltd. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. All experimental procedures carried out in this study were approved by the Animal Ethical and Welfare Committee of Sinovet (Jiangsu) Biopharm. Co., Ltd.

Vaccine preparation: The whole-virus inactivated vaccine was prepared as follows: SW/AH/17 and SW/JS/17 were used as the vaccine strain, the harvested viruses cultured in MDCK were inactivated by inactivated with binary ethyleneimine (BEI; Sigma, USA) and confirmed by inoculating an aliquot of the BEI-treated viruses into MDCK to verify that the cell fluids were negative for hemagglutination. Then, the inactivated viruses were emulsified in adjuvant at a ratio of 45:45:10(SW/AH/17: SW/JS/17: GEL 02 PR).

Vaccination and challenge experiments: A total of 20 4-5-week-old piglets were used in this study. Prior to vaccination, piglets were confirmed to antibody negative for SIVs by use of a hemagglutinin inhibition (Hl)assay and antigen negative for SIVs by use of RT-PCR. One group contained 10 piglets that were vaccinated twice (with two-week interval) with 2ml of vaccine by intramuscular injection. Another group included 10 piglets that received the same volume of phosphate-buffered saline (PBS) as a challenge control. Two weeks after vaccination, serum samples were collected from the vaccine-immunized and PBS-inoculated piglets for HI. To evaluate the protective efficacy of the vaccine against vaccine virus strain SW/AH/17 and SW/JS/17, and epidemic virus strains SW/GD/21 and SW/SD/22, the two groups of 20 piglets were each randomly divided into four subgroups (n=4) and intratracheal injection challenged SW/GD/21 and SW/SD/22 at two weeks after the boost vaccination. After the challenge, clinical symptoms and temperature were observed daily. On day 3-5 post-challenge nasal swabs were collected for virus shedding detection. On day 5 post-challenge all experimental animals were dissected to observe the degree of pathological damage of the lungs.

Serological tests: Sera from immunized piglets were treated with kaolin before being tested for the presence of Hl antibody following international standards (WHO Global Influenza Surveillance Network, Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza). The vaccine virus strain SW/AH/17 and SW/JS/17, and epidemic virus strains SW/GD/21 and SW/SD/22, which represent different antigenic H1N1 and H3N2 viruses, were both used as antigens in the Hl tests.

Virus shedding detection: The filtered and sterilized nasal swab fluid was inoculated into the allantoic cavity of SPF chicken embryos at a dose of 0.2mL per embryo, and then placed in an incubator at 37.5 °C and 50% humidity for further cultivation. The chicken embryos that died after 24h of culture and those that had been cultured for 72h were placed at 4°C overnight, and the chicken embryo allantoic fluid was collected under aseptic conditions. The hemagglutination titer (HA) of chicken red blood cells in the allantoic fluid was detected by micro-hemagglutination assay. When HA≥1:8, indicated that there is virus shedding from piglets, while HA<1:8, indicated that there is no virus shedding from piglets.

Statistical analysis: Antibody titers were compared by use of the two-sided t-test. HI antibody titers detected at same time point using two different antigens were compared. $P<0.05$ was a statistically significant difference, while $P<0.01$ was considered to be an extremely significant difference.

RESULTS

Antibody detection: To evaluate the immunogenicity of the inactivated vaccine, sera were collected from vaccine-immunized and PBS-inoculated piglets two weeks after the boost vaccination, which were analyzed by use of the HI tests. No antibody was detected in the serum samples from the unvaccinated control group. Hl antibody was detected two weeks after the boost vaccination, using SW/AH/17,

SW/JS/17, SW/GD/21 and SW/SD/22, as detection antigens. Hl antibody titers of not less than 1:640 were detected in all immunized piglets, using vaccine virus strain as the antigen. Additionally, HI antibody against epidemic virus strains was also detected at a titer of not less than 1:160. The HI antibody titer of vaccine virus strains used as antigen detection is significantly higher than that of epidemic virus strains (Table 1).

Observation of piglets after challenge: After the challenge of the epidemic virus strains SW/GD/21, three piglets in the unvaccinated control group developed fever, and the maximum temperature exceeded 40.0°C. Four piglets developed respiratory symptoms such as nasal discharge, sneezing or coughing. In the vaccinated group, only one piglet had fever and nasal discharge, while the other piglets had normal temperature and no obvious respiratory clinical symptoms (Fig. 1A, Table 2). After the challenge of the epidemic virus strain SW/SD/22, only two piglets in the unvaccinated control group developed fever, but all five piglets developed respiratory symptoms such as nasal discharge, sneezing or coughing. The body temperature of the five piglets in the vaccinated group was normal and no obvious respiratory symptoms (Fig. 1B, Table 2).

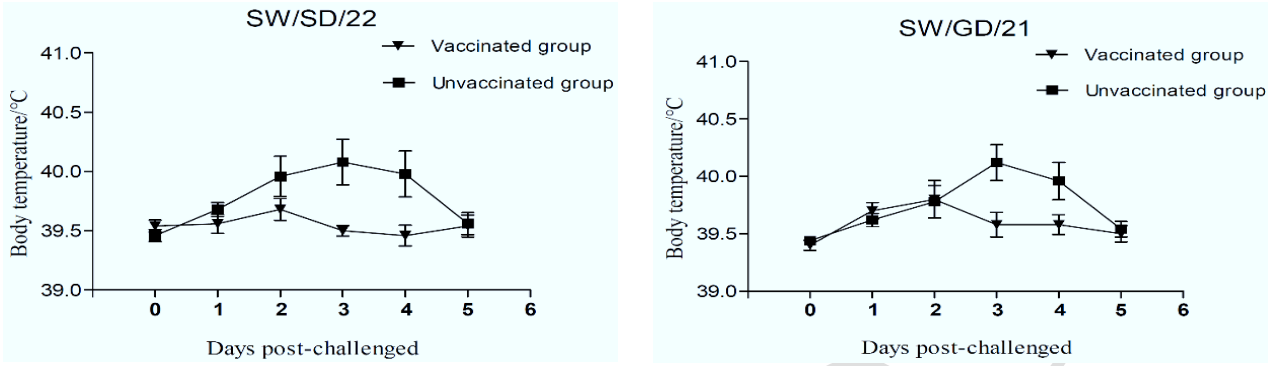

Fig. 1: Temperature of piglets after challenge. The temperature of vaccinated and unvaccinated piglets after challenge SW/GD/21 (A), the temperature of vaccinated and unvaccinated piglets after challenge SW/SD/22 (B).

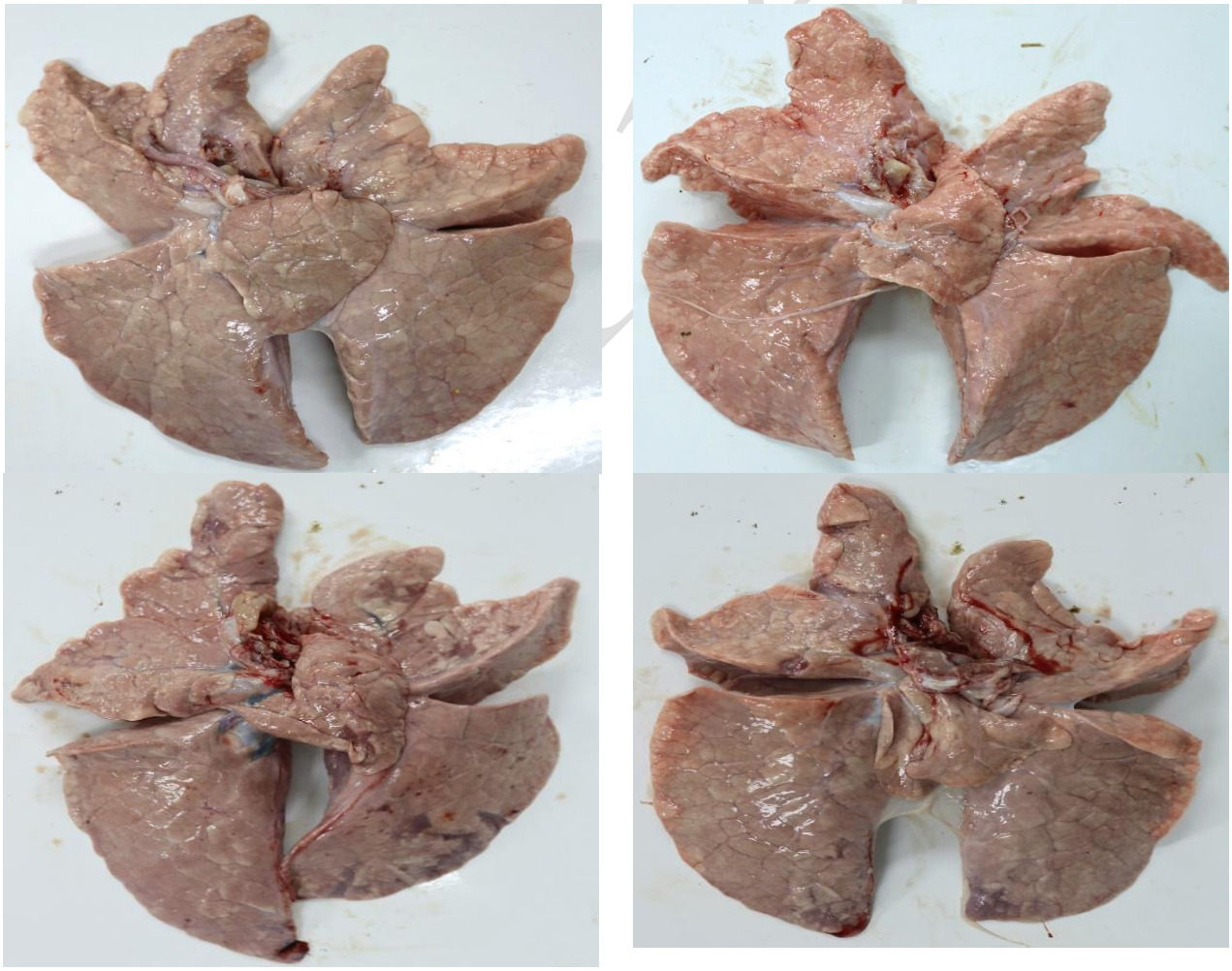

Fig. 2: Macroscopic lung lesions in infected pigs. Photographs of macroscopic lung pathology shown at 5 dpc. The lung of vaccinated piglets after challenge SW/GD/21 (A), the lung of vaccinated piglets after challenge SW/SD/22 (B), the lung of unvaccinated piglets after challenge SW/GD/21 (C), the lung of unvaccinated piglets after challenge SW/SD/22 (D). Pathologica changes, circled in red.

Virus shedding of piglets after challenge: Virus shedding was further measured in nasal swabs specimens collected from pigs during 3-5 dpc. As shown table 3, in vaccinated groups, the inactivated vaccine could provide effective protection, and no virus was detected in nasal swabs specimens of nine piglets. In contrast, nine piglets of unvaccinated group demonstrated virus shedding in the nasal swabs specimens.

Table 1: Detection result of HI antibody in piglet serum after boost vaccination

Group	Antigens detection	for HI antibody titer ($1:10\times2^x$)										T-test
		n1	n2	n3	n4	n5	n6	n7	n8	n9	n10	
Vaccinated	SW/AH/17	6	7	7	6	7	7	6	7	6	6	P=0.0004
	SW/GD/21	4	6	6	5	6	6	5	5	4	5	
	SW/JS/17	8	7	8	7	7	7	7	8	8	7	P=0.0002
	SW/SD/22	6	5	7	6	6	6	7	7	6	6	

Table 2: Clinical symptoms of piglets after the challenge

Group	No.	Days post challenge of SW/GD/21						No.	Days post challenge of SW/SD/22					
		0	1	2	3	4	5		0	1	2	3	4	5
	n1	/	/	/	/	/	/	n6	/	/	/	/	/	/
	n2	/	/	/	c	c	/	n7	/	/	/	/	/	/
Vaccinated	n3	/	/	/	/	/	/	n8	/	/	/	/	/	/
	n4	/	/	/	/	/	/	n9	/	/	/	/	/	/
	n5	/	/	/	/	/	/	n10	/	/	/	/	/	/
	n11	/	/	cd	c	c	/	n16	/	/	/	d	d	d
	n12	/	e	/	d	cd	d	n17	/	/	/	c	c	/
Unvaccinated	n13	/	/	/	/	/	/	n18	/	/	/	d	c	/
	n14	/	/	d	cd	cd	c	n19	/	d	d	/	d	/
	n15	/	/	/	d	d	d	n20	/	/	/	/	c	c

Note: a – Depression, b –Decreased appetite, c –Nasal discharge, d – Sneezing, e –Coughing, / –Absent.

Table 3: Virus shedding of piglets after challenge

Group	No.	Days post challenge of SW/GD/21				No.	Days post challenge of SW/SD/22			
		0	3	4	5		0	3	4	5
	n1	–	–	–	–	n6	–	–	–	–
	n2	–	+	+	–	n7	–	–	–	–
Vaccinated	n3	–	–	–	–	n8	–	–	–	–
	n4	–	–	–	–	n9	–	–	–	–
	n5	–	–	–	–	n10	–	–	–	–
	n11	–	+	+	–	n16	–	+	+	+
	n12	–	+	+	–	n17	–	+	–	–
Unvaccinated	n13	–	+	–	+	n18	–	+	+	+
	n14	–	+	+	+	n19	–	–	–	–
	n15	–	+	+	–	n20	–	+	+	–

Note: "+" Virus was detected, "–" No virus was detected.

Lung pathology of piglets after challenge: Macroscopic lesions of the lungs were observed at 5 dpc. The vaccinated groups were well protected against SW/GD/21 and SW/SD/22, and no obvious pathological changes were found (Fig. 2A, 2B). However, the unvaccinated group contains obvious pathological changes (Fig. 2C, 2D). Histopathologic examination of lungs showed no severe inflammation of bronchioles in the control group (Fig. 3A, 3B), the infected group showed large-area substantiation, and the alveolar structure was blurred (Fig. 3C, 3D).

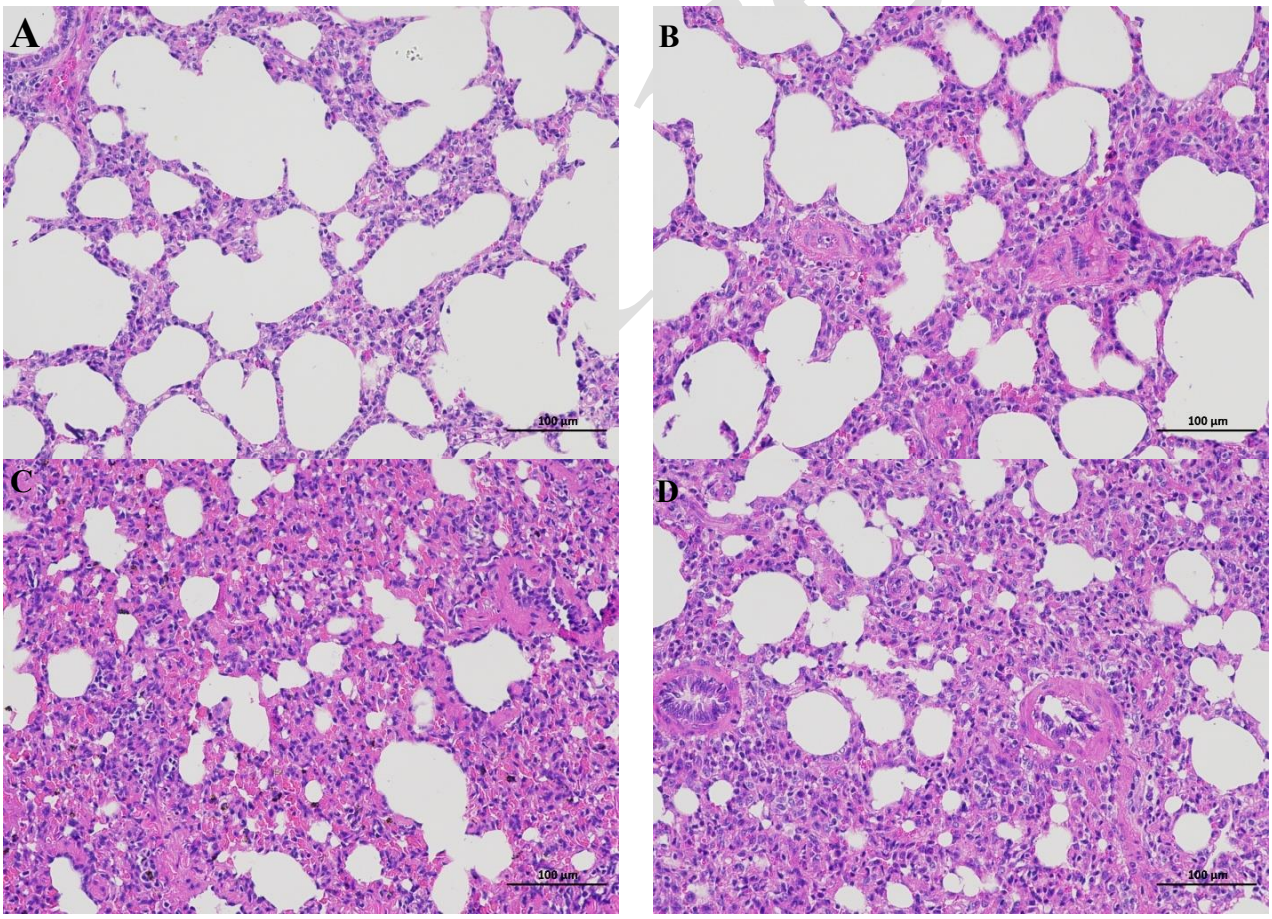

Fig. 3: Histopathologic examination of lung lesions in infected pigs. The lung lesions were observed from four test groups: The lung of vaccinated piglets after challenge SW/GD/21 (A), the lung of vaccinated piglets after challenge SW/SD/22 (B), the lung of unvaccinated piglets after challenge SW/GD/21 (C), the lung of unvaccinated piglets after challenge SW/SD/22 (D). A portion of lung from pigs infected with virus at 5 dpc was fixed 10% phosphate-buffer formalin and processed for paraffin embedding. Each group of samples was stained with haematoxylin and eosin, and examined for histopathological changes (Scale bar = 100μm).

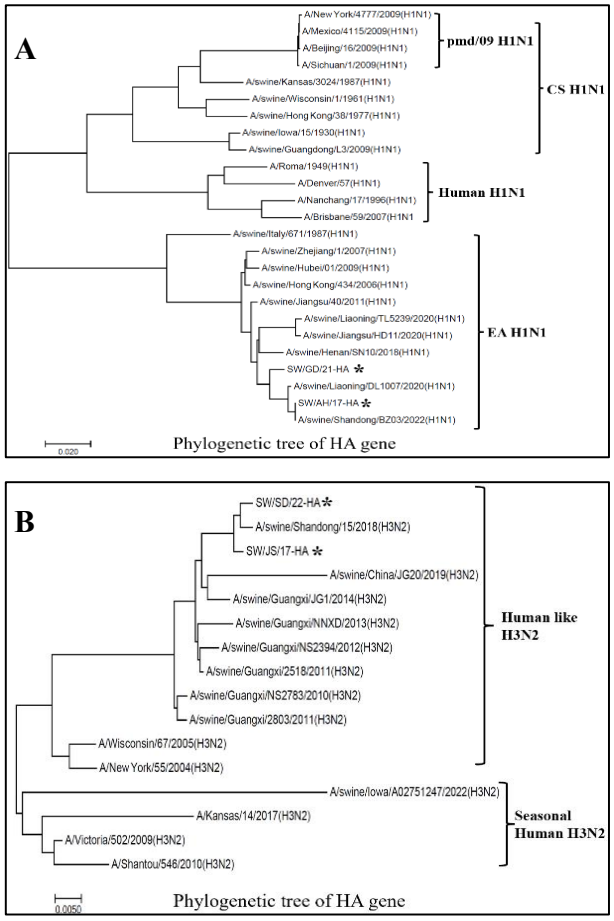

Fig. 4: Genetic Phylogenetic Tree of HA Gene of SW/AH/17, SW/JS/17, SW/GD/21 and SW/SD/22. SW/AH/17 and SW/GD/21 belong to EA H1N1 lineage(A), SW/JS/17 and SW/SD/22 belongs to human-like H3N2 lineage(B).

DISCUSSION

Porcine respiratory tract contains avian SAa-2.3Gal receptor and human SAa-2.6Gal receptor, and considered to be the "mixer" of influenza virus. Avian and human influenza viruses are easy to recombine in pigs to produce new strains, and then cross the host barrier to infect people and cause epidemics, posing a major threat to public health. Many countries, regions and China have reported that SIVs can infect people and cause disease or even death (Dawood *et al.,* 2012; Jhung *et al.,* 2013). SI has important public health significance. Countries all over the world attach great importance to the prevention and control of SI. Vaccination is one of the most effective and economic measures to prevent and control SI. H1N1 subtype and H3N2 subtype SIVs are the main circulating strains in the pig population. Therefore, the research of SIV vaccine is also mainly focused on these two subtypes. At present, a variety of H1N1 subtype, H1N2 subtype or H3N2 subtype monovalent or bivalent inactivated SIV vaccines have been commercially available in some countries (Tang., 2023). Due to the genetic and antigenic characteristics of SIV vary greatly in different regions and countries, the protective effect of commercial vaccines on mismatched subtype strains is reduced, and the vaccine strains need to be continuously monitored and updated (Gauger *et al.,* 2014; Zhao *et al.,* 2024; Zhang *et al.,* 2025).

At present, the prevalent subtypes of SIV are mainly H1N1 and H3N2 subtypes, among which the Eurasian avian H1N1 and human-like H3N2 subtypes have been widely prevalent in Chinese pig farms and formed a stable genetic lineage (Sun *et al.,* 2020; Cai *et al.,* 2022). Previous studies have shown that the strain of the vaccine used in this experiment, SW/AH/17 belongs to the Eurasian avian H1N1 SIV, and SW/JS/17 belongs to human H3N2 SIV. Although the epidemic strain SW/GD/21 used for the challenge belongs to the Eurasian avian H1N1 SIV, and SW/SD/22 belongs to the human-like H3N2, the genetic evolution analysis of the HA and NA genes of the four viruses shows that the epidemic strain is in a different sub branch of genetic evolution from the vaccine strain (Fig. 4A, 4B).

Although HI tests cannot completely accurately detect antibodies in the serum that have neutralizing effects on the virus, HI antibody testing is still commonly used to monitor the effectiveness of influenza vaccination (Gauger *et al.,* 2014). In this study, two types of viruses were used as antigens to detect HI antibodies in immunized animals. Due to genetic and antigenic differences between vaccine strains and epidemic strains, the HI antibody titer detected in epidemic strains was lower, but still higher than the HI antibody qualification standard. The challenge protection test is the best way to evaluate the immune protection effect of vaccines. According to the effectiveness testing method for swine influenza vaccines (Lu., 2019), after challenging epidemic strains SW/GD/21 and SW/SD/22, piglets immunized with swine influenza bivalent inactivated vaccine (H1N1 AH strain + H3N2 JS strain) did not experience fever or respiratory symptoms, reducing viral shedding and reducing lung damage. The results showed that the vaccine still had good protective effect against epidemic strains.

Conclusions: The immunization with swine influenza bivalent inactivated vaccine (H1N1 AH strain + H3N2 JS strain) has good protective effects against epidemic strains SW/GD/21 and SW/SD/22, effectively alleviating the clinical symptoms of infected animals, preventing detoxification, and preventing pathological damage caused by the virus to pigs, which is beneficial for preventing and controlling the current swine influenza virus epidemic in pig herds.

Competing interests: The authors declare that they have no competing interests. All researchers and their affiliated company declare that there are no conflicts of interest associated with this study. The entire study strictly complies with research ethics guidelines, ensuring the objectivity of the results.

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Authors contribution: SC, CC and WS conceived and designed the study. CC, YZ, WL, CX and YS executed the experiment and analyzed the clinical samples. SC and CC analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents.

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