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Original Article

Protective efficacy of a high-growth reassortant swine H3N2 inactivated vaccine constructed by reverse genetic manipulation

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Novel reassortant H3N2 swine influenza viruses (SwIV) with the matrix gene from the 2009 H1N1 pandemic virus have been isolated in many countries as well as during outbreaks in multiple states in the United States, indicating that H3N2 SwIV might be a potential threat to public health. Since southern China is the world's largest producer of pigs, efficient vaccines should be developed to prevent pigs from acquiring H3N2 subtype SwIV infections, and thus limit the possibility of SwIV infection at agricultural fairs. In this study, a high-growth reassortant virus (GD/PR8) was generated by plasmid-based reverse genetics and tested as a candidate inactivated vaccine. The protective efficacy of this vaccine was evaluated in mice by challenging them with another H3N2 SwIV isolate [A/Swine/Heilongjiang/1/05 (H3N2) (HLJ/05)]. Prime and booster inoculation with GD/PR8 vaccine yielded high-titer serum hemagglutination inhibiting antibodies and IgG antibodies. Complete protection of mice against H3N2 SwIV was observed, with significantly reduced lung lesion and viral loads in vaccine-inoculated mice relative to mock-vaccinated controls. These results suggest that the GD/PR8 vaccine may serve as a promising candidate for rapid intervention of H3N2 SwIV outbreaks in China.

Keywords: H3N2 subtype, protective efficacy, reverse genetics, swine influenza virus

Introduction

Since July 2011, a variant influenza A (H3N2) (hereafter, "H3N2v") virus outbreak has been occurring in multiple U.S. states, with 307 confirmed cases, 16 hospitalizations and 1 death (CDC, 12 October 2012). The H3N2v virus contains a 2009 pandemic H1N1 virus (pdm/09) matrix protein segment (pM) and seven genome segments from swine lineage triple-reassortant H3N2 viruses that are circulating in the domestic swine population [2.3]. Novel SwIV that possess the pM have been identified in many countries [6,16,30], including China [6,16]. The M gene of pdm/09, which distinguishes the H3N2v viruses from prior H3N2 SwIV, may have contributed to enhancement of the transmission potential [15]. Recent studies showed that the M gene of pdm/09 promotes aerosol transmission in both a ferret model [13] and a guinea pig model [5]. During late 2011, a total of 12 human infections with H3N2v were identified across five US states. Six of the infected individuals had no history of recent exposure to swine, suggesting the potential for human-to-human transmission, which was confirmed in ferret studies [19]. In addition, H3N2 type viruses tend to cause more severe illness, and higher rates of hospitalization and death were shown in elderly individuals during seasons in which H3N2 subtypes were circulating [27,28].

In a recent study to assess age-related vulnerability to emerging H3N2v infections, researchers found that all children <5 years old and $>80\%$ of children up to 14 years old lack seroprotection, while young adults ($14\sim40$ years old) showed seroprotection [25]. These results are markedly different from prepandemic serosurvey estimates for the pdm/09 virus, to which broad susceptibility across all age groups except very old individuals was identified [1,9]. An important lesson we learned from the 2009 pandemic H1N1 outbreak is that domestic pigs could serve as "mixing vessels" for the genesis of human pandemic strains with gene segments of swine origin [8,26]. The pdm/09 virus harbors ancestral genes, suggesting that the reassortment of swine lineages

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may have occurred years before emergence in humans [26]. Owing to the short life span of pigs, the human influenza virus transmitted to pigs undertake less selection pressure than their counterparts in humans [21]. As a result, pigs have been considered a natural reservoir for older human H3N2 influenza viruses [12]. The increasing antigenic separation between human and swine viruses has made domestic pigs a potential source of pandemic human influenza virus. The recent outbreaks of H3N2v underline the importance of more intensive surveillance and vaccine development for influenza viruses in domestic pigs.

The pdm/09 virus was isolated in domestic pigs one month after the outbreak [11]. Similarly, Influenza A virus subtype H3N2 emerged in humans during the 1968 Hong Kong influenza pandemic, and was then transmitted to domestic pigs [23]. These viruses subsequently circulated in pigs, but occurred infrequently until 1998; however, a swine-adapted human H3N2 virus was transmitted to pigs and rapidly spread throughout the US swine population. Phylogenetic analysis indicated that the hemaggulutinin (HA) of H3N2v descended from the human H3N2 strain that circulated in the mid-1990s; notably, the vaccine reference strain A/Wu han/359/1995 (H3N2), which shares 90% similarity with H3N2v in its HA1 [24]. The outbreaks of H3N2v indicate that human H3N2 ancestor stains were transmitted to domestic pigs during the mid-1990s. Thus, reassortants that were modified in pigs pose a great threat to humans [6].

China is the world's largest producer of pigs, and southern China has been assumed to be an epicenter of pandemic influenza viruses throughout history [22]. In that regard, surveillance and control of swine influenza in China is essential to limiting the possibility of transmission of modified SwIV from swine to humans. Indeed, a number of H3N2 SwIV and H3N2v viruses have been isolated from pigs in China [6,31]. Considering that vaccination with recent seasonal trivalent inactivated influenza vaccine (TIV) does not improve seroprotection [25], vaccines against H3N2 subtype SwIV should be developed to cut the transmission chain of the influenza virus. Egg-grown inactivated influenza virus vaccines have been used widely for humans for many years all over the world, and an ideal seed virus for vaccine production is a strain that is well-matched with the prevailing virus and can also grow well in eggs, which is crucial to mass production. However, naturally isolated viruses usually cannot grow to high titers in eggs, which limits the ability for naturally isolated viruses to serve as seed viruses. Plasmid-based reverse genetics, which was developed in the late 1990s, has become a powerful tool for generation of ideal reassortant influenza vaccine candidates [7,10,18]. In this study, one high-growth H3N2 subtype reassortant influenza virus, GD/PR8 [the HA and NA genes from A/Swine/Guangdong/164/06 (H3N2) (GD/06) virus and six internal genes from the high-growth A/Puerto Rico/8/34(PR8) virus], was generated by plasmid-based reverse genetics and tested as a candidate inactivated vaccine. The immunogenicity and protective efficacy of this vaccine was evaluated in a mouse model.

Materials and Methods

Viruses

A/Swine/Guangdong/164/06 (H3N2) (GD/06) and A/Swine/Heilongjiang/1/05 (H3N2) (HLJ/05) were

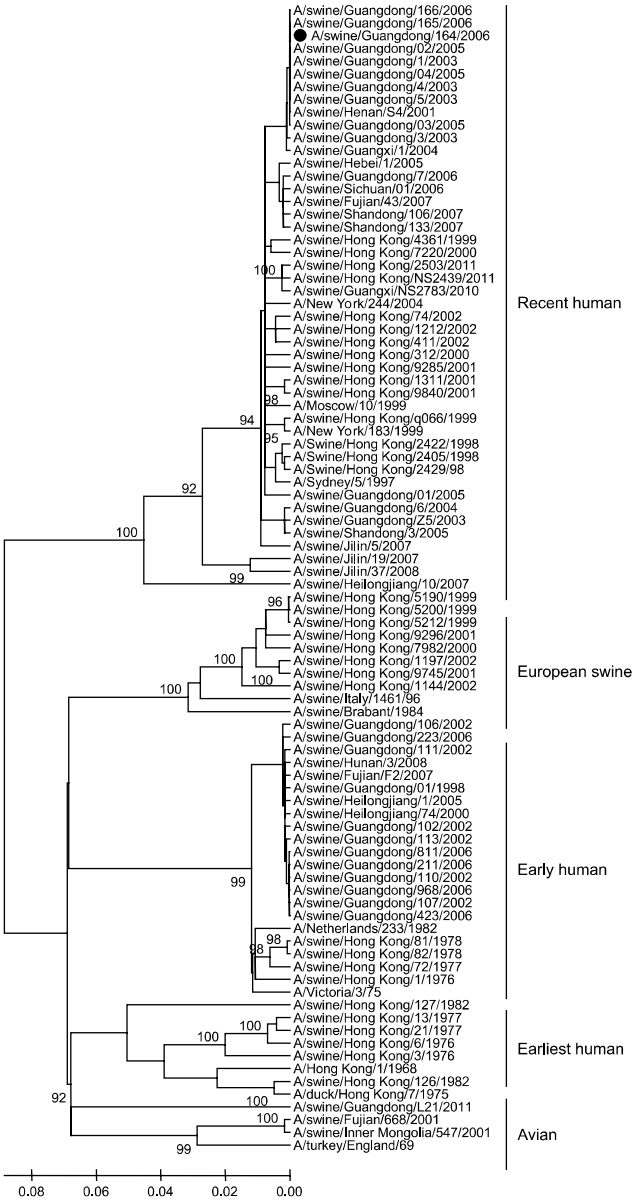

Fig. 1. Phylogenetic analysis of hemaggulutinin (HA) gene segment of H3N2 SwIV isolates from southern China. The HA donor of the seed virus in this study is indicated by a dark circle.

isolated from pig farms during surveillance studies in Guangdong and Heilongjiang province, China, respectively [31]. The phylogenetic analysis of HA gene segment of HA donor of the seed virus (GD/06) in this study is shown in Fig. 1. GD/PR8 was generated by plasmid-based reverse genetics. Specific pathogen-free (SPF) chicken embryonated eggs were used for isolation of viruses, propagation and titration assay. Viruses were kept in a −70°C freezer until the challenge study. All experiments involving H3N2 SwIV were conducted using biosafety level 2 procedures.

Plasmids construction and virus rescue

A 12-plasmid system (8vRNA of all the genome fragments and 4mRNA expression plasmids of PB2, PB1, PA and NP of the PR8 virus) and a bi-directional transcription plasmid pBD were used to generate influenza in this study. The full-length of the HA gene and NA gene of GD/06 were amplified by RT-PCR and inserted into the *BspQ* I site of pBD.

One microgram of each plasmid was added into 250 μL Opti-MEM (Invitrogen, USA) and vortexed. Sixteen microliters of the transfection reagent Lipofectamine 2000 (Invitrogen) were then added into 250 μL Opti-MEM and mixed gently. Five minutes later, the diluted transfection reagent was mixed with the diluted plasmids. The DNA-transfection reagent mixture was kept at room temperature for 20 min and then added directly to a monolayer of 293T cells in a 6-well plate (Costar; Corning, USA). After 6 h of incubation at 37°C in 5% CO_2, the medium was replaced with 2 mL fresh Opti-MEM, and the plate was then incubated as described above for 48 h. The supernatant was subsequently inoculated into the allantoic cavity of 10-day-old embryonated SPF eggs. The allantoic fluid was harvested after 48 h of incubation at 37°C, after which the virus was identified by hemagglutination assay. Reverse transcription PCR (RT-PCR) and re-sequencing confirmed that the genome of the rescued virus was identical in sequence to the cDNA in the plasmids used for its rescue.

Growth kinetics of reassortant virus (GD/PR8)

After three passages of allantoic fluid in embryonated eggs, the growth properties of the reassortant GD/PR8 virus were determined in 10-day-old SPF embryonated eggs as previously described [29].

Preparation of vaccines

Monovalent experimental vaccine was prepared from harvested allantoic fluid with inactivation by formalin (F.I.). Briefly, the virus was inactivated by adding 0.1% formalin (v/v) and kept at 37°C for 48 h. Inactivation of the virus was confirmed by the absence of detected infectivity after two blind passages of formalin-treated allantoic fluid in embryonated eggs. The inactivated GD/PR8 virus was then purified by density gradient ultracentrifugation as previously described [17]. The concentrated virus was subsequently diluted in phosphate-buffered saline (PBS) to 10 μg/50 μL and then emulsified with an equal volume of Freund's complete adjuvant (FCA; first immunization) or Freund's incomplete adjuvant (FIA; booster immunization). One dose (100 μL) of the vaccine contains 10 μg of the GD/PR8 antigen.

Vaccination experiment in mice

Seventy six-week-old SPF female BALB/c mice were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Sixty of these mice were randomly divided into three groups (n = 20 per group), while ten remained untreated as environmental controls. After being allowed to acclimate to their new environment for one week, mice were inoculated with one dose of previously prepared vaccine (GD/PR8+F) or 10 μg concentrated GD/PR8 virus (GD/PR8). Mock-vaccinated mice received 50 μL FCA as a challenge control. All inoculations were administered twice by the multi-point route subcutaneously with a two week interval. All mice were challenged with 10^6 EID_{50} of HLJ/05 virus 14 days post booster immunization. Fifteen mice from each group were euthanized at 4 days post infection (dpi) and the whole lungs were collected for viral RNA detection. Blood samples were collected each week after the first immunization.

All experimental protocols involving mice were approved by the Chinese Ministry of Agriculture and the Review Board of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Mice were handled delicately to avoid any unnecessary discomfort or pain.

Serological assays

ELISA assays to detect total IgG antibodies specific to GD/06 present in serum were performed as previously described [14], with slight modification. Briefly, GD/PR8 virus was purified by density gradient ultracentrifugation and resuspended in PBS, pH 7.8, then diluted to an antigen concentration of 2 μg/mL. Next, the immulon-2HB 96-well plates were coated with 100 μL of antigen solution (200 ng antigen per well) and incubated at 4°C overnight. Mice serum was diluted 50-fold, then subjected to 2-fold serial dilution in PBS. All assays were performed on each sample in duplicate, the means of duplicate wells were calculated and antibody titers were designated as the highest dilution with an OD greater than two standard deviations above the mean of the NV/NC Group (non-vaccinated, non-challenged negative controls). Log_{10} transformations were analyzed and geometric mean reciprocal titers reported.

A hemagglutination inhibiting (HI) assay was performed

according to the World Organization for Animal Health manual. For the serum neutralization (SN) assay, sera were heat-inactivated at 56°C for 30 min, then serially diluted 10-fold in PBS. Subsequently, 100 EID_{50} GD/06 virus was added to each dilution and incubated at 37°C for 1 h. Next, 100 μL of the serum and virus mixture was inoculated into 10-day-old embryonated SPF eggs (3 eggs for each dilution) *via* the allantoic cavity. The allantoic fluid was then harvested after 48 h and verified by Hemagglutination assay, and titers were recorded as the highest dilution. The results were calculated using the method described by Reed and Muench [20].

Viral replication

The presence of virus in the lungs and brains of mice was titered in eggs. Briefly, each sample was 10-fold serially diluted in PBS, after which 0.1 mL aliquots of different dilutions were inoculated into 10-day-old embryonated SPF eggs via the allantoic cavity. The allantoic fluids was harvested after incubation at 37°C for 72 h and tested for hemagglutinin activity. The titer of virus in each sample was calculated by the method described by Reed and Muench [20]. Meanwhile, total RNA was extracted from 300 μL of lungs homogenate (500 μL in total) and the viral loads in the lungs were tested by real-time PCR (RT-PCR). Briefly, cDNA (20 μL) was synthesized using the Uni12 primer: 5′-AGCAAAAGCAGG-3′. RT-PCR was then conducted to evaluate the RNA level using the following primer set: 5′-GACCGATCCTGTCACCTCTGAC-3′ (sense) and 5′-AGGGCATTCTGGACAAAGCGTCTA-3′ (antisense primer), with the TaqMan probe: FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ. The cDNA (1 μL) was used as the template. The reaction consisted of 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 55°C for 30 sec. The M segment of GD/06 was cloned to pMD18-T and served as the standard sample. This sample was subjected to tenfold serial dilution to generate a standard curve. The results were expressed as Log_{10} copies/μL.

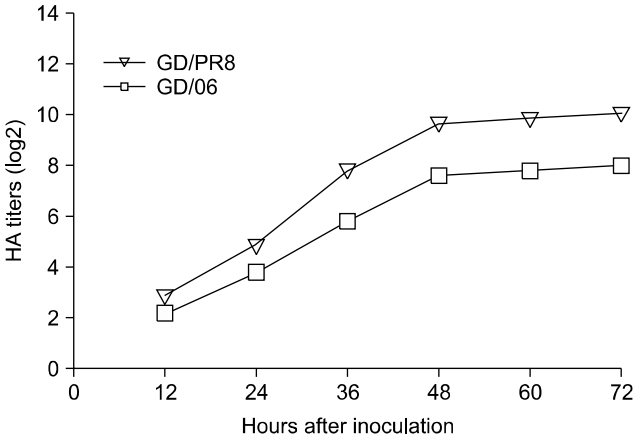

Fig. 2. Growth of the reassortant GD/PR8 virus in embryonated eggs. 0.1 mL of 100EID_{50} of GD/PR8 or GD/06 viruses were inoculated into the allantoic cavities of 10-day-old embryonated eggs and HA titers were checked at 12, 24, 36, 48, 60 and 72 h post-inoculation.

Histopathological analyses

Following 24 h of fixation in 10% formaldehyde, inflated lung samples were embedded in paraffin, cut into 5 μm sections, and mounted on glass slides. Hematoxylin and eosin (H&E) staining was then performed for pathologic examination. A single pathologist reviewed the histopathology in a blinded fashion.

Statistical analysis

Differences in HI and SN antibody titers, ELISA titers and viral loads in the lungs between groups were identified by analysis of variance (ANOVA), with a p value ≤ 0.05 considered to indicate significance (GraphPad Prism; GraphPad Software, USA). Response variables shown to have a significant effect were subjected to pair-wise comparisons using the Tukey-Kramer test. Comparisons were made between each treatment group at each time point using a 5% level of significance ($p < 0.05$) to assess statistical differences.

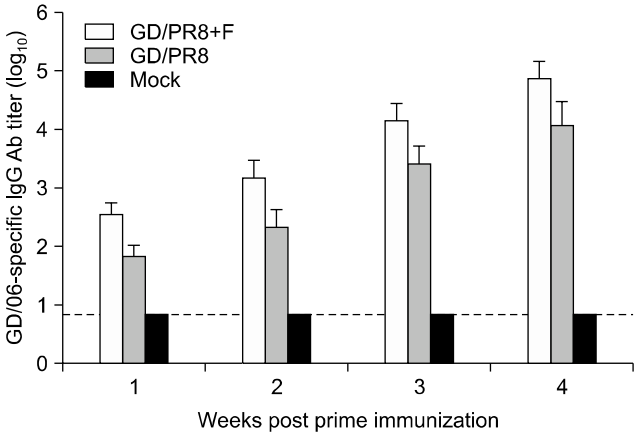

Fig. 3. IgG antibody responses induced by GD/PR8 inactivated vaccines in mice. Sixty 6-week-old SPF female BALB/c mice were randomly divided into three groups (n = 20 per group). Groups of mice were inoculated with one dose of previously prepared vaccine (GD/PR8+F) or non-adjuvanted GD/PR8 virus (GD/PR8) using the same amount of concentrated GD/PR8 virus (10 μg). Mock-vaccinated mice received 50 μL Freund's complete adjuvant (FCA) as a placebo. All inoculations were administered by the muti-point subcutaneous route twice with a two week interval. Serum samples (n = 10 each group) were collected weekly after immunization. All serum samples were assayed for GD/06-specific IgG antibody titers. The results are shown as the mean ± standard deviation for each of the groups of 10 serum samples. Asterisks indicate statistically significant differences ($p < 0.05$) compared with values for mock-vaccinated control mice. The horizontal broken line represents the detection limit.

Results

Growth properties of the reassortant virus GD/PR8

The rescued virus was identified by hemagglutination assay and confirmed by RT-PCR and re-sequencing. The growth properties of the GD/PR8 virus were determined in 10-day-old SPF embryonated eggs after three passages of allantoic fluid in embryonated eggs. All embryonated eggs survived for 72 h after inoculation. Evaluation of the HA titers at different time points revealed that the reassortant GD/PR8 reached 1,024 at 48 h after inoculation (Fig. 2).

Immune responses to vaccine

The immunogenicity of the vaccine was determined in a mouse model. The ELISA assay, HI assay and SN assay were carried out as previously described. For mice that received GD/PR8 inactivated vaccine, IgG antibodies specific to GD/PR8 were detected one week after the first vaccination. The IgG antibody titers were dramatically increased after the booster immunization and reached 10^5 before the challenge. Geometric mean IgG antibody titers are reported in Fig. 3.

The sera samples of the vaccinated group collected at 2 and 4 weeks exhibited significantly higher ($p \leq 0.05$) HI titers and SN titers than the mock group (Table 1). The titers of HI antibodies and SN antibodies against GD/06 in groups of mice are shown in Table 1. The geometric mean HI and IgG antibodies titers reached 2^8 and 10^3 in the GD/PR8+F group at two weeks after the booster immunization.

Table 1. Titers of the mice serum hemagglutination inhibiting antibodies and serum neutralization antibodies against GD/06*

Group†	HI antibody titers		SN antibody titers	
	Weeks post the primary vaccination			
	2	4	2	4
GD/PR8+F	256‡	1024‡	102.8 ± 0.19‡	103.83 ± 0.42‡
GD/PR8	160‡	512‡	101.96 ± 0.23‡	103.22 ± 0.15‡
Mock	< 10	< 10	< 1	< 1

*Serum samples collected at 2 and 4 weeks post primary vaccination were analyzed for HI antibody titers and serum neutralization titers against GD/06 virus. †GD/PR8+F, mice received one dose of vaccine at 7 weeks of age; GD/PR8, mice received one dose of non-adjuvanted GD/PR8 virus; Mock, mice received 50 μL FCA as a challenge control. All inoculations were administered by the muti-point subcutaneous route twice with a two week interval. ‡Significantly different than mock controls. HI: hemagglutination inhibiting, SN: serum neutralization.

SwIV isolation from organs

In the mouse model, the virus loads of lung homogenates [10% (W/V) in PBS] were titrated in eggs as described above. The results of hemagglutinin activity detection and RT-PCR showed that no virus was present in the lungs of challenged mice vaccinated with GD/PR8 vaccine or non-adjuvanted GD/PR8 virus at 4 dpi (Fig. 4). Viral replication was detected in the lungs of all mock-vaccinated

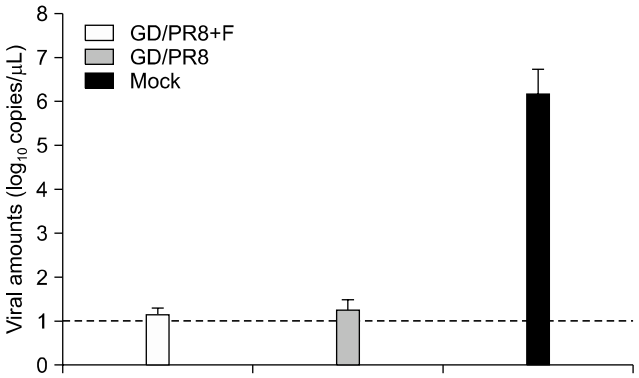

Fig. 4. Detection of viral RNA in lungs of infected mice using real-time PCR (RT-PCR). Copy numbers in $\log_{10}$ per 1 μL of cDNA obtained by RT-PCR targeting the matrix protein gene are given for individual mice. cDNA (20 μL) was synthesized using Uni12 primer by reverse transcription PCR. Asterisks indicate statistically significant differences ($p < 0.05$) compared with values for mock-vaccinated control mice. The horizontal broken line represents the detection limit.

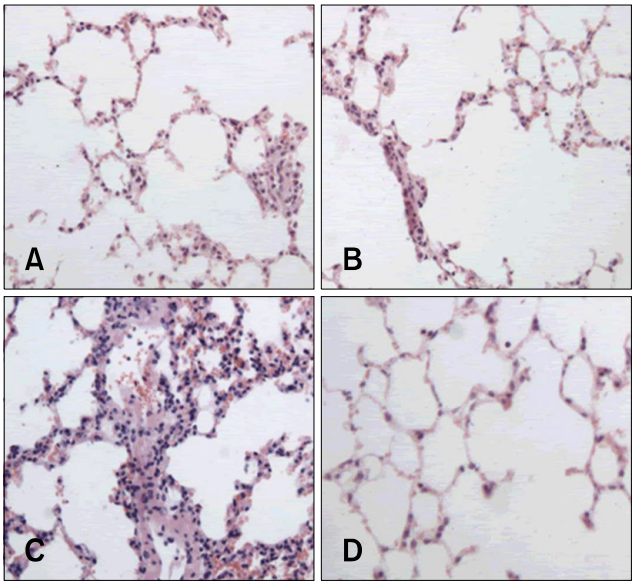

Fig. 5. Microscopic lung lesions in lungs of infected mice. (C) Mock-vaccinated mice with enhanced pneumonia compared to mice immunized with (A) GD/PR8 vaccine and (B) non-adjuvanted GD/PR8 virus. (D) NV/NC mice remained untreated as environmental controls. H&E stain, ×200.

mice, and the virus titers reached $10^{2.20\pm0.27}EID_{50}$/mL, which was significantly higher ($p \leq 0.05$) than the vaccinated groups.

Histopathology

Microscopic lesions in lungs of mice inoculated with 10^6 EID_{50} of HLJ/05 virus at 4 dpi were examined by hematoxylin and eosin (HE) staining. Photomicrographs of HE stained lung sections are shown in Fig. 5. Mice in the mock group displayed prominent histopathological changes in the lungs characterized by acute, diffuse, necrotizing bronchitis and bronchiolitis and pulmonary edema on day 4 post-challenge (Fig. 5C). Mice in the GD/PR8+F (Fig. 5A) and GD/PR8 (Fig. 5B) groups were well protected from the virus and indistinguishable from the NV/NC environmental controls (Fig. 5D).

Discussion

Human pandemic viruses can be transmitted back to domestic swine populations and remain undetected for long periods of time. For example, the influenza H3N2 virus transmitted to domestic pigs after the 1968 Hong Kong pandemic remained relatively inconspicuous until recent years. Serosurveys showed that children < 10 years old lack seroprotection and have little immunity against H3N2v [25], which implies the possibility for a pandemic if H3N2v becomes transmissible among humans. Thus, there is an urgent need to survey and control H3N2 SwIV in domestic pigs. Vaccination is the most efficient means of controlling influenza virus infection. Despite advancements in vaccine efficacy and safety, only inactivated SI vaccines have been approved for clinical use. In this study, we searched for a prevailing H3N2 SwIV as the HA donor of the candidate seed virus. We then applied plasmid-based reverse genetics to generate a high-growth seed virus. We reported the efficacy of the inactivated vaccine in a mouse model.

One isolate, A/Swine/Guangdong/164/06 (H3N2) (GD/06), which has a nucleic acid sequence of HA with high homology to the novel H3N2 SwIVs recently isolated in China, was isolated from one of the main pig production provinces of China. The viral major surface glycoprotein, HA, mediates virus binding and entry into host cells and is the primary target of commercial vaccines. The production of protective antibodies against the main antigenic driver, HA, is crucial to immune protection. Here, the predominant H3N2 SwIV GD/06 was selected as the HA donor to generate the high-growth reassortant virus in this study. However, the GD/06 virus could not replicate efficiently in the mouse respiratory tract. Thus, we chose to challenge mice with A/Swine/Heilongjiang/1/05 (H3N2) (HLJ/05) virus, which can substantially replicate in mice. Sequencing and alignment analysis showed that the nucleic acid sequence of HA of HLJ/05 was 90.9% homologous with that of GD/06 [31]. GD/06 virus matched the prevailing virus well; however, it could not grow to high titers in chicken eggs, which limits its ability to serve as the seed virus. Plasmid-based reverse genetics have been applied to generate high-growth seed virus [4]. The GD/PR8 generated in this study could replicate much more efficiently in eggs when compared with parent virus GD/06, with a titer of 1024HAU/50 μL. All embryonated eggs survived at 72 h after inoculation. The immunogenicity and efficacy of a F.I. vaccine with GD/PR8 as the seed virus was evaluated in a mouse model.

The results of the present study indicated that a single dose of GD/PR8 vaccine or non-adjuvanted GD/PR8 virus could induce HI, SN and IgG antibodies in mice. The specific IgG antibody, which was maintained at a high level over a 4-week period, reached its highest value at 4 weeks post primary immunization. These results showed that the GD/PR8 vaccine could induce a high and long-lasting level of IgG antibody response. High HI, SN and IgG antibodies dramatically increased after the second dose of vaccine. Higher antibody titers were observed in the adjuvanted group compared with non-adjuvanted group. Although HLJ/05 could substantially replicate in mice, it did not cause obvious macroscopic lesions. All groups of mice survived 14 days after the challenge, and none showed significant bodyweight loss relative to the environmental controls. Thus, in this study, we primarily evaluated GD/PR8 as the seed virus by examining the level of specific antibodies and viral loads in the lungs. The mock group that was immunized with FCA had high levels of virus in their lungs. The GD/PR8 vaccine significantly inhibited virus multiplication in the lungs of the adjuvanted group and non-adjuvanted group. Protection against viral replication and pathological changes were evident in mice.

In conclusion, the results of the present study indicate that the reassortant GD/PR8 virus could serve as an ideal seed virus for preparation of an inactivated vaccine to provide the best protection for the swine population and limit potential outbreaks of H3N2v in China.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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