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ORIGINAL ARTICLE

Efficacy of a high-growth reassortant H1N1 influenza virus vaccine against the classical swine H1N1 subtype influenza virus in mice and pigs

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Abstract Swine influenza (SI) is an acute, highly contagious respiratory disease caused by swine influenza A viruses (SwIVs), and it poses a potential global threat to human health. Classical H1N1 (cH1N1) SwIVs are still circulating and remain the predominant subtype in the swine population in China. In this study, a high-growth reassortant virus (GD/PR8) harboring the hemagglutinin (HA) and neuraminidase (NA) genes from a novel cH1N1 isolate in China, A/Swine/Guangdong/1/2011 (GD/11) 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 seed virus for the preparation of an inactivated vaccine. The protective efficacy of this vaccine was evaluated in mice and pigs challenged with GD/11 virus. Prime and boost inoculation of GD/PR8 vaccine yielded high-titer serum hemagglutination inhibiting (HI) antibodies and IgG antibodies for GD/11 in both mice and pigs. Complete protection of mice and pigs against cH1N1 SIV challenge was observed, with significantly fewer lung lesions and reduced viral shedding in vaccine-inoculated animals compared with unvaccinated control animals. Our data demonstrated that the GD/PR8 may serve as the seed virus for a promising SwIVs vaccine to protect the swine population.

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Introduction

Influenza A virus, a member of the family *Orthomyxoviridae*, has a negative-sense, segmented RNA genome and undergoes frequent antigenic shift or reassortment. The emergence of the 2009 novel pandemic influenza A/H1N1 (pH1N1) virus raised wide public concern about SI, and the gene constellation of pH1N1 is a unique combination from swine influenza A viruses (SwIVs) of North American and Eurasian lineages that are circulating in the domestic swine population [6, 23]. Since porcine lung epithelial cells possess receptors for both avian and mammalian influenza virus, pigs have been considered the "mixing vessel" for the generation of new recombinant strains with pandemic potential [6, 10, 14, 20]. Thus, it is of great importance to prevent pigs from H1N1 subtype swine influenza virus infection, which would be likely to cut the transmission chain of influenza virus and greatly reduce the likelihood that a new pandemic influenza virus will be generated.

The first H1N1 subtype SwIV was isolated in the USA in 1930, and it is thought to be a descendant of the 1918 pandemic influenza virus [21]. This H1N1 SwIV and closely related viruses are designated classical H1N1 (cH1N1) SwIVs, and they are currently still circulating in swine populations worldwide [14]. There are three types (H1N1, H3N2 and H1N2) of influenza A viruses consistently isolated from pigs in China [1, 28, 30]. H1N1 is one of the main SwIV subtypes, and cH1N1 viruses remain predominant in China [4, 18]. Only the cH1N1 virus was isolated from US swine prior to 1998, with the exception of one isolation of human H3N2 virus from pigs in Colorado [17]. Unlike the descendants of the 1918 virus circulating in humans, with considerable antigenic drift in HA, the HA antigenicity of the cH1N1 virus is static in the swine

population [7]. The relative stability of the antigenicity of HA in the swine population makes it feasible to develop inactivated vaccines to control the cH1N1 virus.

Vaccination is the most efficient way to control SwIVs infection. Inactivated influenza A vaccines, which became commercially available in 1994, are commonly used in the US swine industry and have played a significant role in preventing clinical disease [2, 11]. China is the largest pig-producing country as well as the largest market for consumption of pork in the world. The number of penned pigs reached 462 million heads in 2012 (http://www.stats.gov.cn). Therefore, it is meaningful to develop efficient vaccines for pigs against these cH1N1 subtypes of influenza virus. Egg-grown inactivated influenza virus vaccines have been used in humans for many years. An ideal seed virus for vaccine production is a strain that is well-matched with the prevailing virus and also can grow well in eggs, which is crucial for mass production [3]. Plasmid-based reverse genetics was developed in the late 1990s and has proved to be a powerful tool to generate ideal reassortant influenza vaccine candidates [5, 8, 13, 16].

In this study, one high-growth H1N1 subtype reassortant influenza virus, GD/PR8 (with HA and NA genes from GD/11 virus and six internal genes from the high-growth PR8 virus) was generated by reverse genetics and tested as a candidate seed virus for the preparation of an inactivated vaccine. The immunogenicity and protective efficacy of this vaccine was evaluated in mice and pigs.

Materials and methods

Viruses

One influenza virus was isolated from pigs in Guangdong Province of China in 2011 and identified as H1N1 SwIV in hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays with a panel of reference antisera recommended by the World Health Organization (WHO) (http://www.who.int/csr/resources/pulications/en/#influenza). This SwIV was designated as A/swine/Guangdong/1/11(H1N1) (GD/11). The reassortant virus (GD/PR8) for the candidate vaccine in this study was generated by plasmid-based reverse genetics [25]. GD/PR8 harbors the HA and NA genes from A/swine/Guangdong/1/11(H1N1) (GD/11) and six internal genes from A/Puerto Rico/8/34(H1N1) (PR8). These viruses were propagated in specific-pathogen-free (SPF) embryonated chicken eggs and titrated to determine the 50 % egg infective dose (EID_{50}) by the method of Reed and Muench [19]. All experiments involving H1N1 SwIVs were conducted using biosafety level 2 procedures.

Generation and growth kinetics of reassortant virus (GD/PR8)

Influenza GD/PR8 virus harboring the HA and NA genes from GD/11 and six internal genes from PR8 was generated by plasmid-based reverse genetics and then confirmed by re-sequencing. The growth properties of the new recombinant GD/PR8 virus were determined in 10-day-old SPF embryonated eggs as described previously [25].

Preparation of vaccines

The monovalent experimental vaccine was prepared from harvested allantoic fluid (containing 1024 HAU/50 μl) and inactivation by formalin treatment. Briefly, the virus was inactivated by adding 0.1 % formalin (v/v) and kept at 37 °C for 48 h. The 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 virus was then emulsified in paraffin oil adjuvant (Hangzhou Oil Refining Company, Hangzhou, China) at a v:v ratio of 1:1.5 virus to adjuvant.

Animal experiments

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

Mouse model

Seventy six-week-old SPF female BALB/c mice were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China), and sixty of them were randomly divided into three groups (N = 20 per group). Ten mice remained untreated as an environmental control. After acclimating to new environment for a week, mice were inoculated with the inactivated GD/PR8 vaccine (GD/PR8 + adjuvant) or non-adjuvanted GD/PR8 virus (GD/PR8) at the same dose of 0.2 ml (containing 1600 HA units). Mock-vaccinated mice received the same volume of phosphate-buffered saline (PBS) as a challenge control. All inoculations were administered twice subcutaneously at multiple sites with an interval of two weeks. All mice were challenged with 0.2×10^5 EID_{50} of GD/11 virus 14 days after the second immunization. Fifteen mice of each group were euthanized at 4 days postinfection (dpi), and whole lungs were collected for detection of viral RNA. Blood samples were collected each week after the first immunization. The survival rate, clinical symptoms and

bodyweight of the mice were monitored for 14 days after the challenge.

Swine experiment

Eighteen three-week-old cross-bred piglets were obtained from a herd that was free of SwIV, classical swine fever virus (CSFV). and porcine reproductive and respiratory syndrome virus (PPRSV). The piglets were antibody negative against H1N1 by Idexx enzyme-linked immunosorbent assay (HerdCheck Swine Influenza Virus (H1N1) Antibody Test Kit, Idexx Laboratories) according to the manufacturer's instructions. The piglets were acclimatized for two weeks. Then, fifteen of them were randomly assigned to three groups (5 piglets per group). Three piglets that were not vaccinated and not challenged (NV/NC) were used as an environmental control. The piglets were separated by group and housed in a specific-pathogen-free room. At 5 weeks of age, the group of one-dose pigs (N = 5) were individually inoculated intramuscularly (i.m.) with 2 ml of vaccine (containing 16,000 HA units) prepared previously once, while the group of two-dose pigs (N = 5) were individually inoculated i.m. with 2 ml of vaccine twice with a three-week interval. The group of mock-infected pigs (N = 5) received the same volume of PBS as a challenge control. Serum samples were collected weekly after the first immunization.

Pigs challenged at 11 weeks of age were inoculated intratracheally with 2 ml of 2×10^5 EID_{50} of GD/11 virus. Pigs were monitored daily for signs of clinical disease, and rectal temperatures were measured. Nasal swabs were taken daily for five days after challenge. At 5 dpi all of the pigs were humanely euthanized, their lungs were examined for lesions, and their viral load was measured. Serum and the following tissues were collected: brain, heart, liver, lung, kidney and spleen.

Serological assays

ELISA assays to detect total IgG antibodies specific for GD/PR8 present in serum were performed as described previously [27] with modifications. Briefly, concentrated GD/11 virus was resuspended in PBS, pH 7.8, and diluted to an HA concentration of 100 HA units/50 µl. Then, the concentrated solution was diluted 1000-fold with PBS, and immulon-2HB 96-well plates were coated with 100 µl of antigen solution (200 HA units per well) and incubated at 4 °C overnight. Mouse serum was diluted 100-fold, followed by twofold serial dilution in PBS. Swine serum collected from all groups was diluted 1000-fold, followed by twofold serial dilution in PBS. The assays were performed on each sample in duplicate. The mean of duplicate wells was calculated, and antibody titers were designated as the highest dilution with an OD greater than 2 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 are reported.

The HI assay was performed according to the World Organization for Animal Health manual (http://www.oie.int/Eng/Normes/Mmanual/2008/pdf/2.08.08_SWINE_INFLUENZA.pdf). Briefly, sera were heat inactivated at 56 °C for 30 min, treated with receptor-destroying enzyme to remove nonspecific hemagglutinin inhibitors and natural serum agglutinins, and centrifuged. Supernatants were then serially diluted in V-shaped-bottom 96-well microtiter plates in an equal volume of 4 HA units of GD/11 virus. Plates were incubated at room temperature for 10 minutes, followed by adsorption with a 0.5 % chicken erythrocyte suspension.

For the serum neutralization assay (SN), sera were heat-inactivated at 56 °C for 30 min, then tenfold serially diluted in PBS. Subsequently 100 EID_{50} of GD/11 virus was added to each dilution and incubated at 37 °C for 1 h. One hundred microliters 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 harvested after 48 h and verified by hemagglutination assay, and titers were calculated by the method of Reed and Muench.

Viral replication and shedding

The presence of virus in the organs and nasal swabs was measured in eggs as described previously [29]. Briefly, each sample was serially diluted tenfold in PBS, and different dilutions were inoculated in a 0.1-ml volume into 10-day-old embryonated SPF eggs via the allantoic cavity. The allantoic fluid was harvested after incubation at 37 °C for 72 h and then tested for hemagglutinin activity. The titer of virus in each sample was calculated by the method of Reed and Muench [19]. Meanwhile, total RNA was extracted from 300 µl of lung homogenate from mice (500 µl in total) and the viral loads in the lungs were tested by real-time PCR. Briefly, cDNA (20 µl) was synthesized using the primer Uni12 (5′-AGCAAAAGCAGG-3′). Real-time PCR was performed to determine the RNA level, using the sense primer 5′-GACCGATCCTGTCACCTCTGAC-3′, the antisense primer 5′-AGGGCATTCTGGACAAAGCGTCTA-3′, and the TaqMan probe FAM-TGCAGTCC TCGCTCACTGGGCACG-BHQ. The cDNA (1 µl) was used as the template. The reaction was performed at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s. The M segment of A/Swine/Guangdong/164/06 (GenBank: EU273779) was cloned to pMD18-T and served as the standard sample. It was

serially diluted tenfold to generate the standard curve. The results were expressed as Log_{10} copies/μl.

Pathologic examination of lungs

At necropsy, lungs were removed and evaluated to determine the percentage of the lung affected with purple-red consolidation typical of SwIV infection. Tissue samples from the trachea and right cardiac lung lobe were taken and fixed in 10 % buffered formalin for histopathologic examination. Tissues were routinely processed and stained with hematoxylin and eosin.

Statistical analysis

Macroscopic pneumonia scores, HI and neutralization antibody titers, ELISA titers, and nasal swab virus titers for different groups were analyzed using analysis of variance (ANOVA) with a p-value ≤ 0.05 considered significant (GraphPad Prism, GraphPad Software). Response variables shown to have a significant effect by treatment group were subjected to pairwise comparisons using the Tukey-Kramer test. Comparisons were made between the treatment groups at each time point using a 5 % level of significance ($P < 0.05$) to assess statistical differences.

Results

Characterization of the reassortant virus GD/PR8

The reassortant GD/PR8 virus, harboring the HA and NA genes from GD/11 and six internal genes from PR8, was generated by plasmid-based reverse genetics and confirmed by sequence analysis. GD/PR8 virus could replicate efficiently in eggs, with a titer of 1024 HAU/50 μl and had an infectivity of 10^7 EID_{50}/ml. The growth properties of the GD/PR8 virus were determined in 10-day-old SPF embryonated eggs, and all embryonated eggs survived at 72 h after inoculation. The HA titers were checked at different time points (Fig. 1). The HA titers of GD/PR8 reached 1024 at 48 h after inoculation and stopped increasing after that (Fig. 1).

Antibody response induced by the GD/PR8 inactivated vaccine in mice and pigs

The immunogenicity of the reassortant GD/PR8 virus vaccine was determined in mice and pigs using the immunization schedules described above. In the mouse model, serum samples collected at 2 and 4 weeks exhibited significantly higher ($P \leq 0.05$) HI titers and serum neutralization titers compared to the control group (Fig. 2).

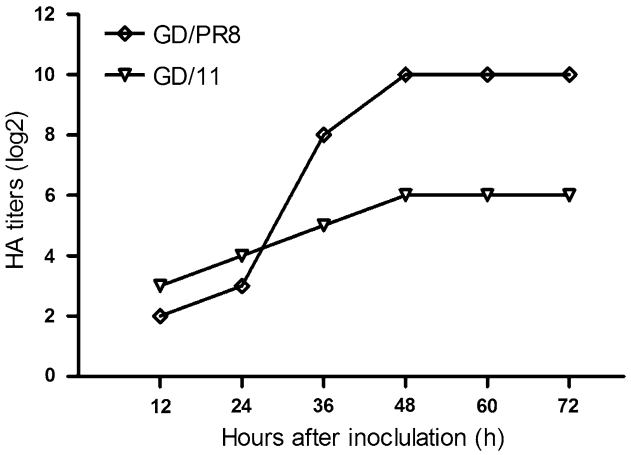


Fig. 1 Growth properties of GD/PR8 and GD/11 viruses in embryonated eggs. 0.1 ml of 100 EID_{50} reassortant virus GD/PR8 or novel isolated virus GD/11 was inoculated into the allantoic cavities of 10-day-old embryonated eggs. The HA titers were determined at 12, 24, 36, 48, 60 and 72 h post-inoculation

The geometric mean IgG antibody titers are shown in Fig. 2C. All pigs tested antibody negative against H1N1 by Idexx enzyme-linked immunosorbent assay prior to the experiment. The HI antibodies and IgG-specific antibodies against GD/11 in pigs vaccinated with GD/PR8 vaccine are shown in Table 1. HI and IgG antibodies were detected in pigs immunized with GD/PR8 vaccine one week after vaccination. High titers of HI antibodies were observed one week after the second immunization and reached the highest level with titers of 1024. In the pigs that received two doses of the vaccine, the IgG antibody levels were dramatically increased after the second immunization and reached the highest level five weeks after the primary vaccination.

SwIV isolation from organs and swabs

In the mouse model, both the results of hemagglutinin activity detection and the results of real-time PCR showed that virus was not detected in the lungs of vaccinated mice at 3 dpi. Viral replication was detected in the lungs of all mock-vaccinated mice, and the mean virus titers reached $10^{3.2}$ EID_{50}/ml, which were significantly higher ($P \leq 0.05$) than in the vaccinated groups (Fig. 3). For the swine experiment, the viral load in nasal swabs and tissue homogenates (10 % [W/V] in PBS) was determined by titration in eggs as described previously. Nasal swabs were collected daily for 5 days after challenge. No virus was detected in the two-dose-vaccinated pigs, and virus was detected in two one-dose vaccinated pigs on the first day after the challenge, while infectious virus was detected in the nasal swabs of all pigs in the mock-vaccinated group from days 1–4 dpi

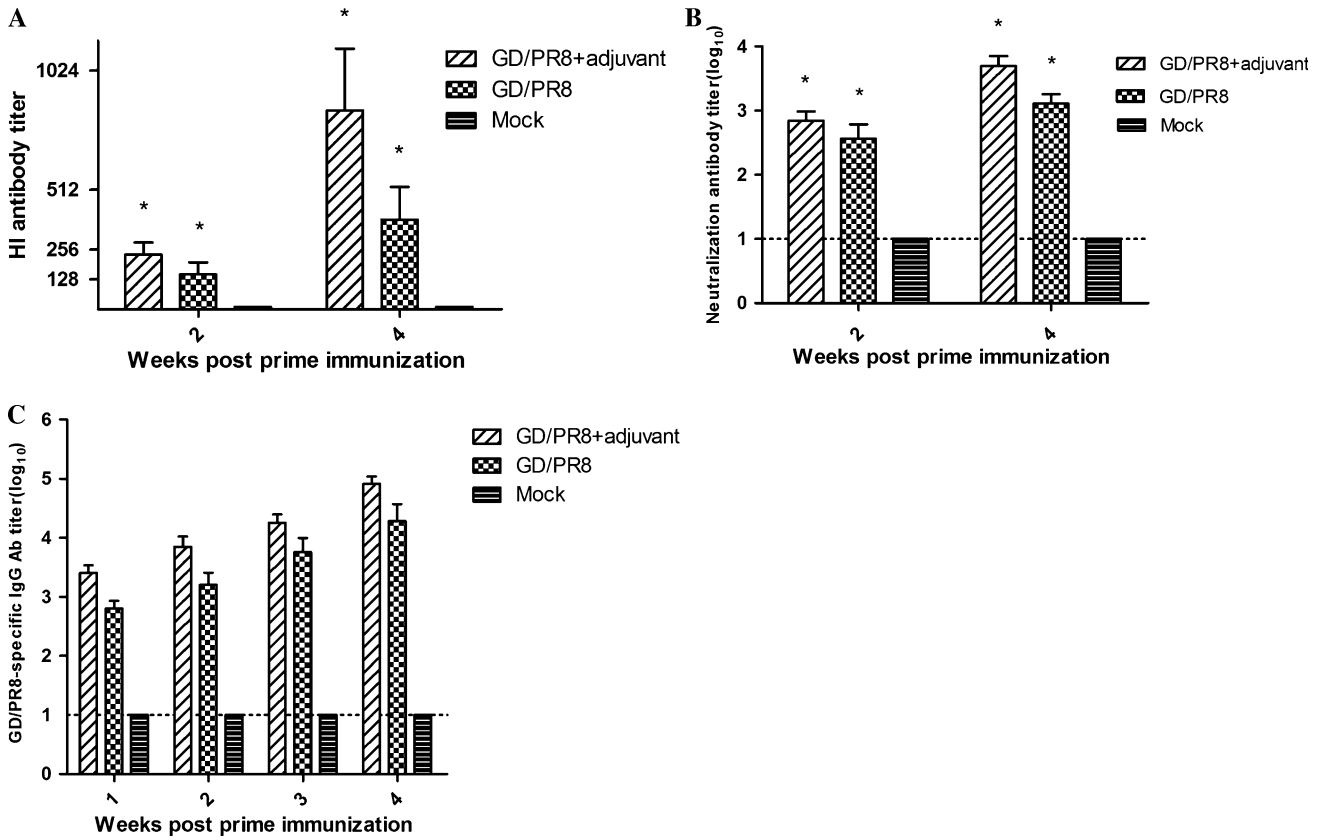


Fig. 2 Antibody responses induced by GD/PR8 inactivated vaccines in mice. Sixty six-week-old SPF female BALB/c mice were randomly divided into three groups (N = 20 per group). Mice were inoculated subcutaneously at multiple sites with a previously prepared vaccine (GD/PR8 + adjuvant) or non-adjuvanted GD/PR8 virus (GD/PR8) at the same dose of 0.2 ml (containing 1600 HA units). Mock-vaccinated mice received 0.2 ml PBS as a placebo. All groups of mice received a booster vaccination with the same dose of vaccine two weeks after the primary vaccination. Serum samples (N = 10 each group) were collected weekly after the immunization. Serum samples were collected at 2 and 4 weeks after the primary vaccination, and HI antibody titers (**A**) and serum neutralization titers (**B**) against GD/11 virus were determined. All serum samples were assayed for rG11-specific IgG antibody titers (**C**). 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

Table 1 Geometric mean titers of swine serum HI antibodies and IgG-specific antibodies against GD/11

Group	Hemagglutination inhibition						IgG ELISA					
	Weeks after primary vaccination						Weeks after primary vaccination					
	1	2	3	4	5	6	1	2	3	4	5	6
One-dose	21	96*	307*	307*	307*	256*	3600*	8000*	8800*	19200*	24960*	16960*
Two-dose	26	102*	307*	922*	922*	819*	3200*	7200*	8800*	38400*	108800*	105600*
Mock	≤10	≤10	≤10	≤10	≤10	≤10	≤1000	≤1000	≤1000	≤1000	≤1000	≤1000

One-dose, group of pigs received one dose of 2 ml inactivated GD/PR8 vaccine at 5 weeks of age; two-dose, group of pigs received one dose of 2 ml inactivated GD/PR8 vaccine and were boosted with the same dose of vaccine three weeks after the primary vaccination; mock, the group of pigs received the same dose of PBS at 5 weeks of age as a challenge control

* Significantly different than mock controls

(Table 2). GD/11 virus was only detected in the lungs of challenged pigs. No virus was detected in any of the organs collected from the NV/NC control pigs (Table 2).

Clinical and pathological changes

In the mouse model, all groups of mice survived after challenge. Mock-vaccinated mice displayed lethargy and

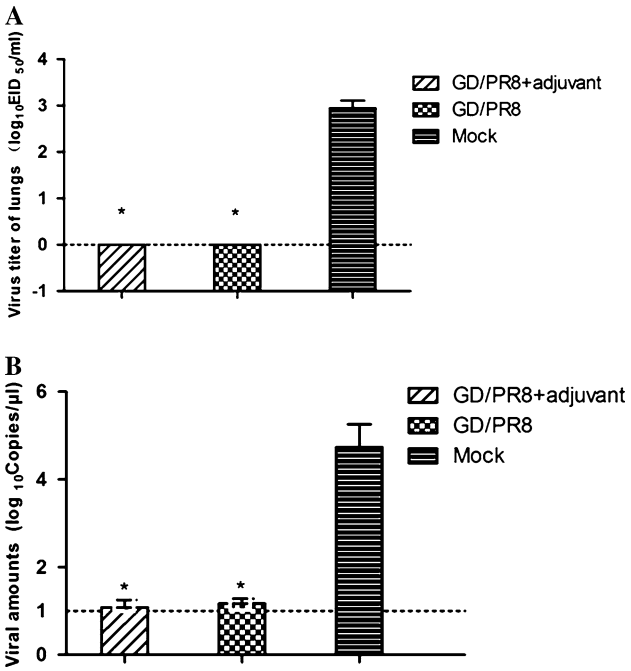


Fig. 3 Changes in mean lung viral titers after homologous virus challenge. Groups of mice were inoculated subcutaneously at multiple sites with a previously prepared vaccine (GD/PR8 + adjuvant) or non-adjuvanted GD/PR8 virus (GD/PR8) at the same dose of 0.2 ml (containing 1600 HA units). Mock-vaccinated mice were immunized with the same volume of PBS. Fifteen mice from each group were euthanized at 3 days post-infection (dpi), and whole lungs were collected for virus RNA detection. Each lung homogenate sample was divided into two parts and titrated in embryonated eggs ($log_{10}EID_{50}$/ml) (Fig. 2A) or by real-time PCR (Fig. 2B). Asterisks indicate statistically significant differences ($P < 0.05$) compared with values for mock-vaccinated control mice; the horizontal broken line represents the detection limit

weight loss (Fig. 4), while the mice vaccinated with the GD/PR8 vaccine or non-adjuvanted GD/PR8 virus lost only a moderate amount of weight during infection in the first two days and then fully recovered any body weight that had been lost (Fig. 4). Mock-vaccinated mice displayed acute, diffuse, necrotizing bronchitis and bronchiolitis and pulmonary edema on day 4 post-challenge (Fig. 5A). Only slight histopathologic changes were observed in the mice of the vaccinated groups (Fig. 5B and C), and they were well protected from GD/11 challenge.

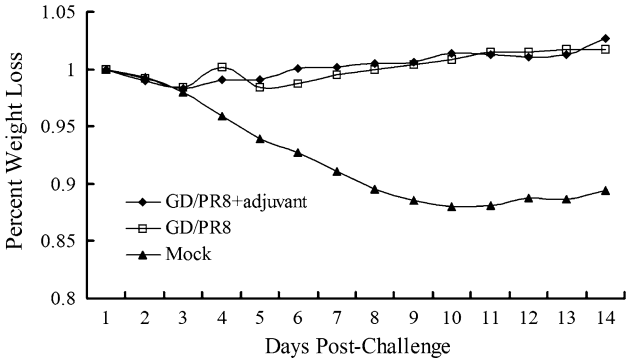


Fig. 4 Changes in mean body weights after homologous virus challenge. Groups of 6-week-old female BALB/c mice were immunized with a previously prepared vaccine (GD/PR8 + adjuvant) or non-adjuvanted GD/PR8 virus (GD/PR8). Mock-vaccinated mice received PBS as a challenge control. Two weeks later, mice were boosted with the same amount of vaccine. Four weeks after the primary vaccination, mice were challenged intranasally with GD/11 virus. Body weight was measured daily after the challenge and is given as mean per group (N = 5)

Table 2 Virus replication in nasal swabs and organs of vaccinated or control pigs

Group	Challenge virus	Virus replication in nasal swabs[a]					Virus replication in organs of pigs on day 5 dpi[b]					
		Days post-challenge										
		1	2	3	4	5	Lung	Liver	Spleen	Kidney	Heart	Brain
One-dose	GD/11	2/5	-	-	-	-	-	-	-	-	-	-
Two-dose		-	-	-	-	-	-	-	-	-	-	-
Mock		5/5	5/5	5/5	5/5	4/5	5/5	-	-	-	-	-
NV/NC		-	-	-	-	-	-	-	-	-	-	-

Groups of five-week-old pigs (N = 5 in each group) were individually inoculated intramuscularly (i.m.) with 2 ml one-dose or two-dose (with a three-week interval) of vaccine or PBS, and then all were challenged with 2 ml of 2×10^5 EID_{50} of GD/11 virus at eleven weeks of age. Group NV/NC (3 pigs) remains untreated as an environmental control

'-' indicates that no virus was detected in the sample

'x/5' indicates that the virus was detected in x of 5 pigs in the group

[a] Nasal swabs were collected for five days after the challenge and the virus level in the nasal swabs was determined in eggs. The allantoic fluids was harvested after incubation at 37 °C for 72 h and then tested for hemagglutinin activity

[b] All pigs form each group were euthanized at five days post-challenge, and the organs were collected for virus detection in eggs

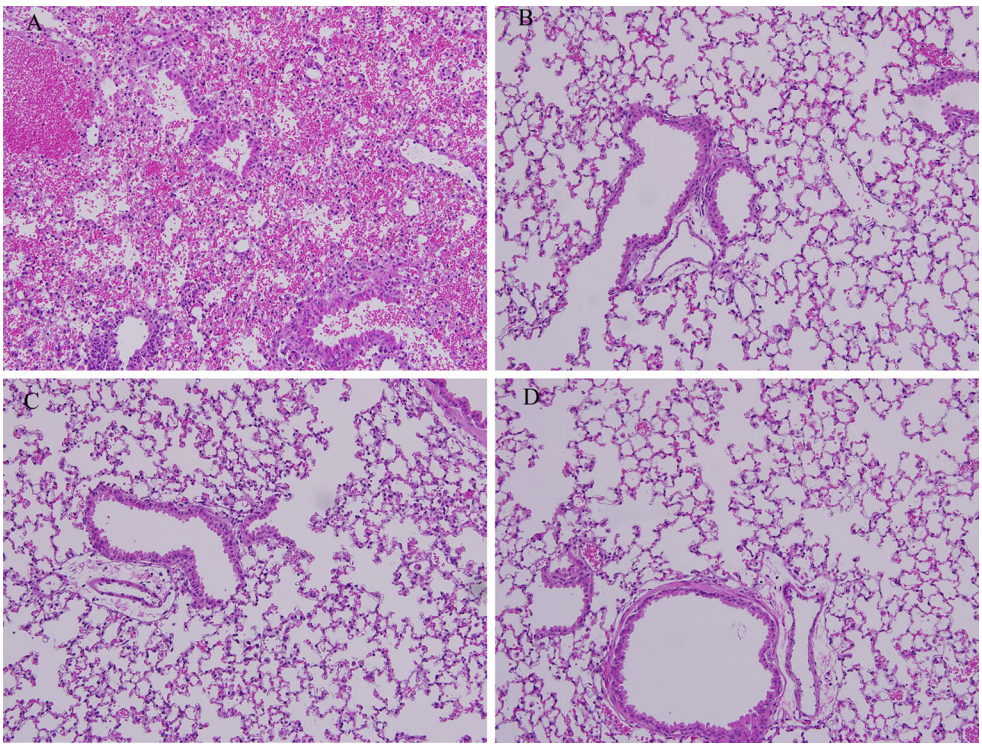


Fig. 5 Microscopic lung lesions representing mice in each challenge group. (**A**) Mock-vaccinated mice with enhanced pneumonia compared to mice immunized with GD/PR8 vaccine (**B**) and non-adjuvanted GD/PR8 virus (**C**). NV/NC mice remained untreated as an environmental control (**D**). Hematoxylin and eosin staining (HE). Magnification, 200×

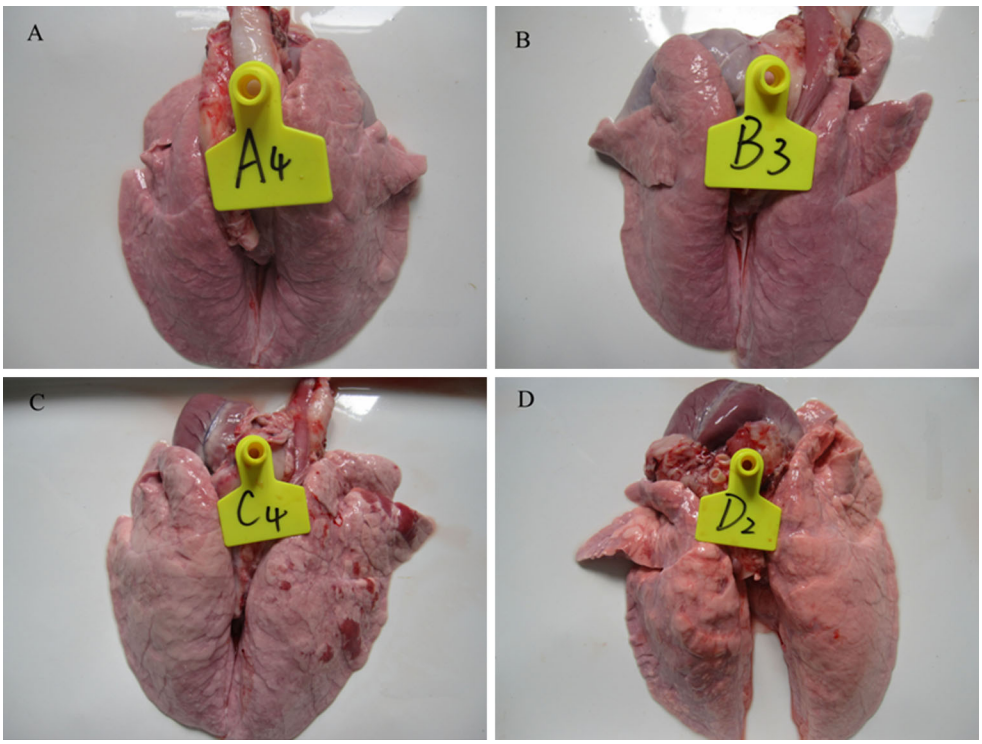


Fig. 6 Macroscopic lung lesions in infected pigs. A few plum-colored, depressed lobules can be observed in the apical and anterior diaphragmatic lobes of mock-vaccinated pigs after challenged with GD/11 virus at 5 dpi (**C**). No lung lesions were observed in GD/PR8-vaccinated pigs (**A**, **B**)

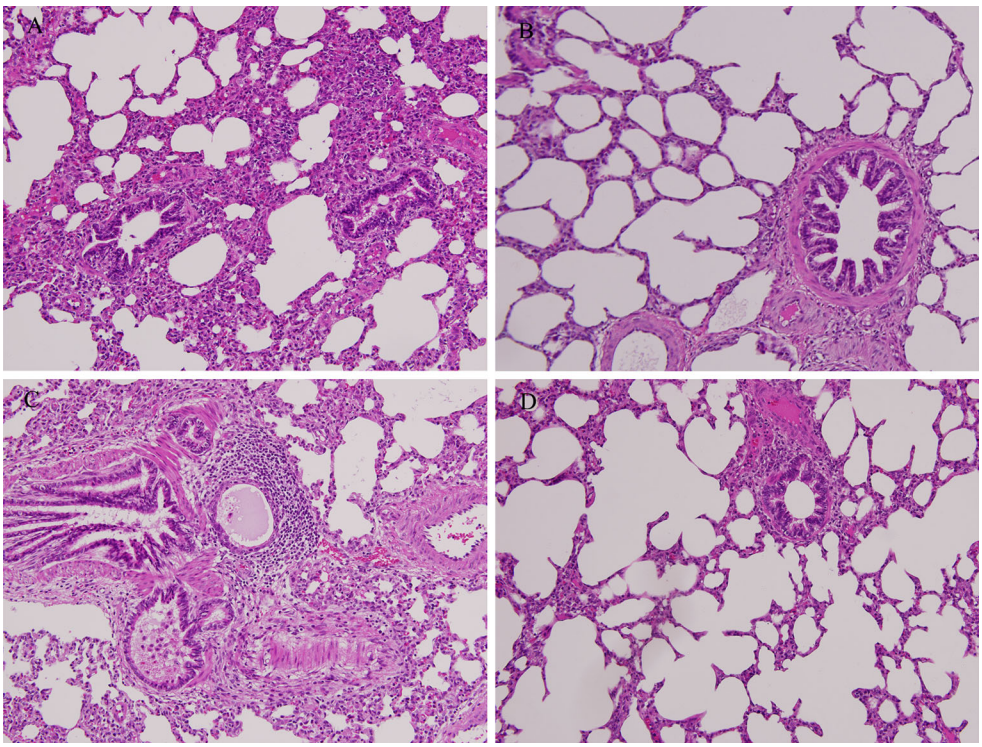


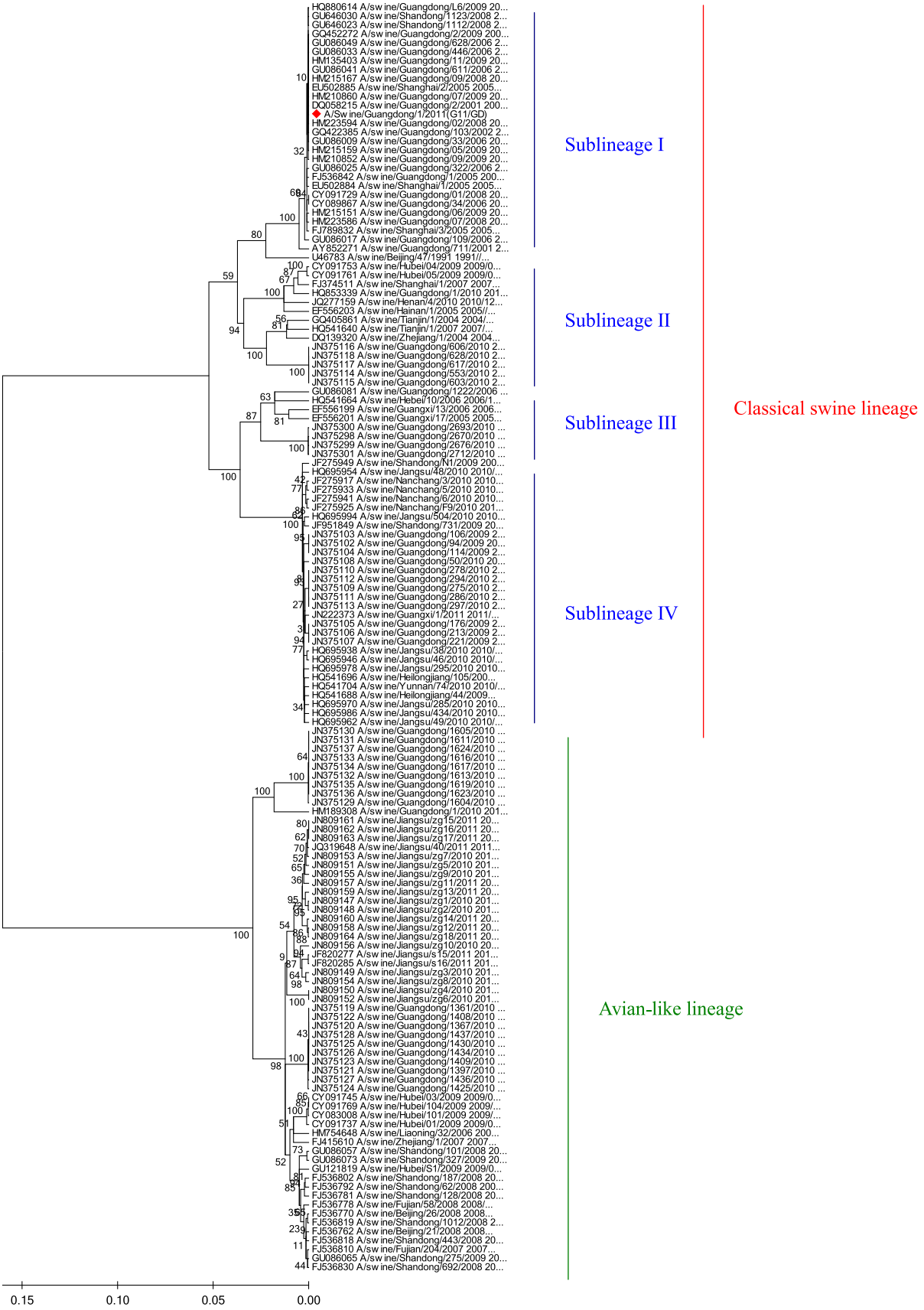
Fig. 7 Microscopic lung lesions in infected pigs. Moderate to severe inflammation of bronchioles characterized by epithelial necrosis and attenuation, neutrophil infiltration, and peribronchiolar cuffing of mononuclear cells was observed in mock-vaccinated pigs (**C**). Mononuclear cell infiltration surrounding small bronchioles was observed in one-dose-vaccinated pigs (**A**), while pigs that received a two-dose vaccine were completely protected from GD/11 virus (**B**). The NV/NC pigs served as an environmental control (**D**). Hematoxylin and eosin staining (HE). Magnification, 200×

The non-vaccinated, non-challenged mice (NV/NC) served as environment control (Fig. 5D).

All pigs were free of influenza A virus and tested antibody negative against H1N1 by Idexx enzyme-linked immunosorbent assay prior to the start of the experiment. Pigs were challenged with GD/11 virus at 11 weeks of age and euthanized at 5 dpi. Clinical signs of disease and rectal temperatures were recorded daily after challenge. Mock-vaccinated pigs developed mild to moderate lethargy, and anorexia, sporadic coughing, and tachypnoea were observed by 2 dpi. No flu-like symptoms were observed in the GD/PR8-vaccinated pigs, and no difference was observed between them and the NV/NC pigs. Rectal temperatures did not change significantly after challenge. None of the GD/PR8-vaccinated or control pigs displayed any clinical signs.

Macroscopic and microscopic lesions of the lungs were observed in the PBS-vaccinated pigs at 5 dpi. Pigs of the one-dose and two-dose groups were well protected from the virus and showed a statistically significant reduction in percentage of animals with macroscopic pneumonia over the mock-vaccinated pigs. A few irregularly distributed, plum-colored consolidated depressions could be observed in the apical and anterior diaphragmatic lobes of mock-vaccinated pigs at 5 dpi (Fig. 6). Histopathologic examinations of the lungs showed that there was moderate to severe inflammation of bronchioles characterized by epithelial necrosis and attenuation, neutrophil infiltration, and peribronchiolar cuffing of mononuclear cells in the mock-vaccinated pigs (Fig. 7C). Mononuclear cell infiltration surrounding small bronchioles was observed in the one-dose vaccinated pigs (Fig. 7A). Moreover, the microscopic lesions of the lungs of the two-dose group were indistinguishable from those of the NV/NC environmental controls (Fig. 7).

Fig. 8 Phylogenetic tree of the HA genes of all of the H1N1 swine influenza isolates in China. The unrooted phylogenetic tree was generated by the distance-based neighbor-joining method using MEGA 3.1. The reliability of the tree was assessed by bootstrap analysis with 1000 replications, and only bootstrap values >90 % are shown. A/Swine/Guangdong/1/2011(GD/11) is indicated by a red diamond. The HA genes of all the swine H1N1 isolates in China could be segregated into two lineages, including a classical swine lineage and an avian-like lineage. The classical swine lineage is further separated into four sublineages, and our isolate GD/11 is located in sublineage I ▶


HQ880614 A/sw ine/Guangdong/L6/2009 20...
GU646030 A/sw ine/Shandong/1123/2008 2...
GU646023 A/sw ine/Shandong/1112/2008 2...
GQ452272 A/sw ine/Guangdong/2/2009 200...
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GU086033 A/sw ine/Guangdong/446/2006 2...
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FJ536770 A/sw ine/Beijing/26/2008 2008...
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GU086065 A/sw ine/Shandong/275/2009 20...
FJ536830 A/sw ine/Shandong/692/2008 20...
0.15
0.10
0.05
0.00
Sublineage I
Sublineage II
Sublineage III
Sublineage IV
Classical swine lineage
Avian-like lineage

Discussion

The viral surface glycoprotein HA plays a critical role in the virus life cycle [22]. The production of antibodies induced by vaccines against HA is crucial for immune protection [7]. Here, we have attempted to develop an HA-based vaccine that has been reported in previous studies [15, 29]. We isolated one strain of SwIV from a pig farm in Guangdong, China. A phylogenetic tree of GD/11 is shown in Fig. 8. Comparison of the nucleic acid sequence of the HA of GD/11 with novel SwIVs isolated recently in China shows high homology, which indicates that GD/11 and closely related virus are currently prevailing in the swine population of China. Therefore, we selected GD/11 as the HA and NA donor to generate the reassortant virus.

It is frequently asked whether vaccines containing older virus strains would protect efficiently against new emerging SwIVs, although SwIVs do not drift as much as human influenza viruses. Research has been conducted on this issue and has shown that a commercial vaccine containing the A/New Jersey/8/76 H1N1 virus could protect pigs efficiently against challenge with an antigenically and genetically different H1N1 field isolate from 1998 [26]. Vaccines with as low as 84 % amino acid sequence homology to the HA1 protein of the challenge virus could still provide adequate virological protection against challenge [24]. SwIV vaccine manufacturers are not obliged to regularly replace their vaccine strains like human and equine influenza vaccines [12].

The seed virus for preparation of an inactivated vaccine should be propagated efficiently in eggs. However, naturally isolated virus usually cannot grow to high titers in eggs, which limits their ability to be used as a seed virus. Plasmid-based reverse genetics has been applied to generate high-growth seed virus [3, 29]. The parent virus GD/11 has a titer of 64 HAU/50 μl and an infectivity of 10^5 EID_{50}/ml, while GD/PR8 generated in this study has a titer of 1024 HAU/50 μl and an infectivity of 10^7 EID_{50}/ml. Therefore, the growth kinetics of these two viruses showed that GD/PR8 grew to much higher titers than GD/11. Thus the reassortant virus generated in this study replicated more efficiently in eggs compared with the parent virus GD/11. The immunogenicity and efficacy of a formalin-inactivated vaccine with GD/PR8 as the seed virus was evaluated in a mouse model prior to the start of the swine experiment.

The efficacy of an oil-emulsion inactivated vaccine for SI has been demonstrated in pigs [29]. Mice and pigs immunized with GD/PR8 vaccine or non-adjuvanted GD/PR8 virus induced high HI, SN and IgG antibody titers against GD/11. These antibodies dramatically increased after the second dose of vaccine. The GD/PR8 vaccine induced moderately higher antibody titers in mice when compared with non-adjuvanted GD/PR8 virus. High virus titers were detected in the lungs of mock-vaccinated mice, while no virus was detected in the mice of the other two groups. Protection against viral replication, weight loss, and pathological changes was evident in mice. This showed that humoral immunity induced by inactivated vaccines can effectively eliminate homologous virus. This is consistent with other human and animal studies [15, 29]. All mock-vaccinated mice survived and lost no more than 15 % body weight, indicating that GD/11 and related cH1N1 SwIVs did not replicate well and had relatively low pathogenicity in mice. This may be due to SwIVs, like human influenza virus, being unable to replicate efficiently in mice without prior adaption to the host. The mock-vaccinated pigs showed only a sporadic cough two days after GD/11 challenge. Rectal temperatures did not change significantly, and no nasal discharge was observed in any of the groups of pigs. Clinical signs, pathologic changes and virus replication were restricted to the respiratory tract. The clinical signs of SI were relatively mild, though virus replicated efficiently in the respiratory tract. These results are consistent with previous studies [29], and the data showed that miniature pigs only exhibited asymptomatic infection after challenge with pH1N1 virus [9]. This suggests that cH1N1 SwIVs can persist in the swine population while causing relatively mild or indistinguishable damage to the pigs. It may help us to understand that the pigs are able to act as a "mixing vessel" from which novel influenza viruses may emerge. Although the severity of swine influenza was not so obvious in the experimental environment, the damage of SI may be aggravated by secondary bacterial infection under natural conditions.

In this study, a high-growth GD/PR8 virus was generated with the currently predominant cH1N1 SwIVs GD/11 and PR8 as its parent viruses. The monovalent inactivated GD/PR8 vaccine provided optimal protection against GD/11 challenge according to all of the parameters that were evaluated. Based on the results demonstrated here, the GD/PR8 vaccine is an ideal vaccine candidate to be used in pigs to provide protection for the swine population and limit the potential reassortment of novel pandemic influenza viruses in the future.

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