Construction of a Bartha-K61-like vaccine using the CRISPR/Cas9 method confers complete protective immunity against emerging PRV variant challenge in piglets

Running title: a Bartha-K61-like vaccine (PRV GDFS-delgI/gE/US9/US2 live-attenuated vaccine)

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ABSTRACT

Pseudorabies virus (PRV) causes Aujeszky's disease or pseudorabies (PR) in pigs worldwide, which leads to heavy economic losses to the swine industry. Since 2011, the emerging PRV variant led to the outbreak of PR in Bartha-K61-vaccinated pigs. The PR outbreaks demonstrated that Bartha-K61 vaccine did not provide full protection against the emerging PRV variant. PRV live-attenuated vaccines could control PRV infection, which has become a consensus. In this study, a Bartha-K61-like vaccine based on emerging PRV variant was generated by the CRISPR/Cas9 method, which has deleted the gI, gE, US9, and US2 genes. Safety experiments have confirmed that PRV GDFS-delgI/gE/US9/US2 was safe for 5-7 days-old suckling piglets. The piglets immunized with PRV GDFS-delgI/gE/US9/US2 vaccine did not produce PRV gE-specific antibodies but could generate PRV gB-specific antibodies and high neutralizing titers against PRV GDFS strain (variant PRV strain) or PRV Ea strain (older PRV strain). After emerging PRV GDFS variant challenge, all piglets immunized with PRV GDFS-delgI/gE/US9/US2 vaccine did not show any clinical signs, and the rectal temperature was normal. Moreover, the autopsy and histopathological analyses revealed that the piglets in the PRV GDFS-delgI/gE/US9/US2 vaccine group did not show apparent gross and pathological lesions. Furthermore, the piglets did not present weight loss in the PRV GDFS-delgI/gE/US9/US2 vaccine groups. According to the criteria of OIE terrestrial manual, the results of the experiment confirmed that the PRV GDFS-delgI/gE/US9/US2 vaccine could provide full protection against emerging PRV variant strain in piglets. Therefore, PRV GDFS-delgI/gE/US9/US2 strain is a potential live-attenuated vaccine against emerging PRV variant strain infection in China.

Keywords: Pseudorabies virus, CRISPR/Cas9 technology, Bartha-K61-like vaccine, Safety

and Efficacy.

1 INTRODUCTION

Pseudorabies virus (PRV) is a member of the family Herpesviridae, subfamily Alphaherpesvirinae, and genus *Varicellovirus* (Sun et al., 2016). PRV causes Aujeszky's disease or pseudorabies (PR) in pigs worldwide. Pigs are the natural host, reservoir, and source of infection. PRV infection causes high mortality in newborn piglets, respiratory symptoms and growth retardation in finishing pigs, and reproductive failure in sows, which lead to heavy economic losses to the swine industry (Leng et al., 2013; Z. Gu, Hou et al., 2015).

Bartha-K61-attenuated vaccine is considered safe and effective and plays important roles in protection against PRV infection(B. Dong et al., 2014). Bartha-K61 is developed by in vitro continuous passage culture, and gene sequencing of Bartha-K61 genome shows that almost 3500 bp of a large fragment in the genome is deleted, including the complete gE and US9 genes and part of gI and US2 genes(B. Dong et al., 2014)(Szpara et al., 2011). Bartha-K61 vaccine is widely used to deracinate PR in the North America and some European countries in the past decades (Freuling et al., 2016).

Bartha-K61 vaccine is imported from Hungary to China in 1979, which is widely used in China and has played a critical role in the control of PR from 1990 to 2010 (Sun et al., 2016). However, since 2011, the outbreaks of infection with the variant PRV have been confirmed in most regions of China (Leng et al., 2013; Y. Wang et al., 2015; J. Gu et al., 2018; Liu et al., 2018; Cheng et al., 2020; Ma et al., 2020). After the PRV epidemic, many PRV-negative pig farms have become positive, which causes significant economic losses to the pig industry. Since the outbreak of variant PRV among Bartha-K61-vaccinated pigs in large-scale pig farms, several studies have shown that the Bartha-K61 vaccine cannot provide full protection against the emerging PRV

variants (Leng et al., 2013; J. Gu et al., 2018). A number of studies have demonstrated that genetic mutation can be observed in the virulent and protective antigen genes of variant PRV genome (Luo et al., 2014a; Li et al., 2017; Yu et al., 2017; Ren et al., 2020). Phylogenetic analysis indicates that the new PRV isolates belong to genotype II, which are different from the classical PRV strains, such as NIA3, Becker, and Kaplan strains (Z. Gu, Hou et al., 2015). Thus, developing a safe and effective vaccine against the emerging PRV variants is necessary. Three variant PRV strains from aborted fetus samples from three pig farms of Bartha-K61-vaccinated pigs were isolated in our laboratory. These variant PRV strains could cause 80%–100% mortality in 50–60 days-old piglets. In this study, we constructed a gI/gE/US9/US2-deleted attenuated vaccine strain on the basis of variant PRV strain (PRV GDFS), referring to the deletion of PRV Bartha-K61 strain by using the CRISPR/Cas9 technology. The safety and effectiveness of PRV GDFS-delgI/gE/US9/US2 vaccine were investigated in a suckling piglet model. PRV GDFS-delgI/gE/US9/US2 has no pathogenicity for suckling piglet and confers complete protection against PRV infection in suckling piglets. Thus, PRV GDFS-delgI/gE/US9/US2 is a potential attenuated vaccine strain against emerging PRV variant infection.

2 MATERIALS AND METHODS

2.1 Virus and Cells

PRV-GDFS (GenBank No. MH521043) was isolated from Guangdong Province of China. PK-15 cells were cultured in DMEM (Gibco, USA) with 10% fetal bovine serum (HyClone, USA) and 5% CO_2 at 37 °C in a humidified incubator.

2.2 Construction of transfer plasmid and sgRNA plasmids

A transfer plasmid was constructed by using two segments flanking the gI and US2 genes (Figure 1). The fragments of gI-L and US2-R were amplified by polymerase chain reaction (PCR)

with gD-F/gI-R and US2-F/US2-R primers. Then, the two PCR products were inserted into the pBluescript II SK (pSK) vector. Finally, the transfer plasmid pSK-gIL-US2R was obtained, as the recombination homologous arms. The pCas9-gI targeting site was 5′-TACGACCCCGCGTCCCCCG-3′, and the pCas9-US2 targeting site was 5′-GGGGTGACGGCCATCACCG-3′. The guide RNAs were synthesized and cloned into the PX335 plasmids. All the sequence of primers and sgRNAs were listed in Table 1.

2.3 Generation of PRV GDFS-delgI/gE/US9/US2 by CRISPR

The homologous recombination and CRISPR technology were used simultaneously to gene-delete virus. Co-transfection was conducted in PK-15 cells using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. In brief, 4 μg of pSK-gIL-US2R plasmid, 8 μg of PRV-GDFS genome, and 1 μg of pX335-sgRNAs (0.5 μg of pX335-gIsgRNA and 0.5 μg of pX335-US2sgRNA) were co-transfected to PK-15 cells as previously described. After the cytopathogenic effect (CPE), the cells were collected and subjected to 3 cycles of freezing and thawing. The PRV GDFS-delgI/gE/US9/US2-deleted virus was generated through plaque purification assay. The recombinant virus was identified by PCR test using gI/US2-F/ R specific primers (Table 1), and the genetic stability was validated by consecutive culture of cells.

2.4 Immunofluorescence assay

PK-15 cells cultured to 90% confluence in 12-well plates were infected with PRV GDFS-delgI/gE/US9/US2 at an MOI of 0.001, and PRV GDFS wild-type strain served as the positive control. Twenty-four hours after the infection, the infected cells were fixed using cold methanol:acetone (1:1), followed by washing with PBS. Then, the cells were blocked in blocking buffer (5% bovine serum albumin in PBS) and incubated with anti-gE mAbs or anti-gB mAbs (1:100 dilution; the monoclonal antibodies were provided by Doctor Bo Hou). After washing three

times with PBS, the cells were incubated with FITC-conjugated goat anti-mouse antibody (1:500 dilution, ABclonal, Wuhan, China). The cells were investigated under a fluorescent microscope (Olympus IX73, Japan).

2.5 Animal experiments

2.5.1 Safety experiment

Twenty 5–7 days-old suckling piglets were purchased from a PRV-negative pig farm. The suckling piglets were confirmed to be seronegative for PRV using the PRV-specific gE and gB antibody ELISA kit (IDEXX, USA) and were randomly divided into four groups of five. The piglets in group A (negative control group) were injected intramuscularly with 1 ml of DMEM. The piglets in group B (positive control group) were injected intramuscularly with a single dose of commercial Bartha-K61 vaccine ($10^5$$TCID_{50}$/Dose). The piglets in group C were injected intramuscularly with 10^5 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2. The piglets in group D were injected intramuscularly with 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2. All pigs were checked daily for rectal temperature, and clinical signs (respiratory symptoms: sneezes, breathlessness, and nasal discharges; neurologic symptoms: opisthotonos and ataxia) were recorded throughout the experiment.

2.5.2 Efficacy experiment

Twenty 5–7 days-old suckling piglets free of PRV antibodies were randomly divided into four groups with five piglets per group. The pigs in group A (negative control group) were injected intramuscularly with 1 ml of DMEM. The piglets in group B (positive control group) were injected intramuscularly with a single dose of commercial Bartha-K61 vaccine (HIPRA, Spain). The piglets in group C were injected intramuscularly with 10^5 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2. The piglets in group D were injected intramuscularly with 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/

US9/US2. All piglets at 28 days post-primary immunization (DPI) were challenged intranasally with $10^{8.0}$ $TCID_{50}$ dose of virulent PRV GDFS strain.

After the PRV challenge, all piglets were checked daily for rectal temperature, and clinical signs (respiratory symptoms: sneezes, breathlessness, and nasal discharges; neurologic symptoms: opisthotonos and ataxia) were recorded throughout the experiment. Body weights of all pigs were individually measured at 0 days post-challenge (DPC) (challenge) and 14 DPC (necropsy). Average weight gain was calculated and analyzed.

2.6 Serological tests

Serum samples were collected at 0, 7, 14, 21, and 28 DPI and tested for the detection of the PRV-specific gB and gE antibodies using the commercialization of PRV ELISA kits (IDEXX, USA), according to the manufacturer's instructions.

The serum neutralization test was performed as described previously. Fifty microliters of serum samples were serially diluted twofold and mixed with 100 $TCID_{50}$ PRV GDFS strain or PRV Ea strain at 37 °C for 60 min. The mixture was added to the confluent PK-15 cells cultured in 96-well plates and then incubated at 37 °C and 5% CO_2 for 4 days. The cells were investigated under a microscope for the CPE. The titers of neutralization antibodies were calculated as the reciprocal of the highest serum dilution that no CPE was observed (Z. Gu, Dong et al., 2015).

2.7 Viral shedding

The nasal swab samples were collected at 0, 2, 4, 6, 8,10,12, and 14 DPC and were clarified through centrifugation. The supernatant samples were passed through a sterile 0.22micron filter and were serially diluted tenfold. Then, the dilutions were inoculated to PK-15 cells on 96 well culture plates. Viral titers of the nasal swab samples were calculated as $TCID_{50}$.

2.8 Necropsy and histopathological examination

At 14 DPC, piglets from each group were euthanized. Complete necropsy of each animal was performed. The samples were collected and fixed in 10% neutral-buffered formalin. Histopathological examination of the tonsils and brain was performed with HE stains.

2.9 Statistical analysis

Statistical analysis was conducted using the GraphPad prism 6.0 (GraphPad Software, USA). One-way ANOVA was used for statistical analyses among different groups. $P < 0.05$ was defined as statistically significant difference.

3 RESULTS

3.1 Generation of the recombinant virus PRV GDFS-delgI/gE/US9/US2

The recombinant virus PRV GDFS-delgI/gE/US9/US2 was constructed by co-transfection with PRV-GDFS genome, pX335-sgRNAs and pSK-gIL-US2R plasmid. At 72 h post-transfection, the cytopathic viruses were collected and purified by three rounds of plaque assay. The purified viruses were identified by PCR assay with specific primers (gI/US2-F and gI/US2-F). A specific 3964 bp fragment covering gI/gE/US9/US2 genes was detected in wild-type PRV GDFS strain, but the 580 bp fragment was identified in recombinant virus, which the gI/gE/US9/US2 genes were deleted (Figure 1). The 580 bp fragment of genes deleted was validated by gene sequencing. Therefore, the purified and identified genes deleted in gI/gE/US9/US2 were named as PRV GDFS-delgI/gE/US9/US2. The recombinant virus PRV GDFS-delgI/gE/US9/US2 was consecutively cultured in PK-15 cells for 20 passages to verify genetic stability. The PCR products and sequencing indicated that PRV GDFS-delgI/gE/US9/US2 was a genetically stable strain after continuous cultivation for 20 generations. The 20th passaged PRV GDFS-delgI/gE/US9/US2 strain was used as vaccine virus seed.

PK-15 cells were infected with recombinant virus PRV GDFS-delgI/gE/US9/US2 strain or

wild-type PRV GDFS strain to clarify the absence of gE gene in the recombinant virus. As shown in Figure 2, PK-15 cells infected with the recombinant viruses showed gB-specific green fluorescence. Moreover, no gE-specific green fluorescence was observed in cells infected with PRV GDFS-delgI/gE/US9/US2. These results indicated that the recombinant virus have deleted the genes of gI/gE/US9/US2.

3.2 Safety experiment of PRV GDFS-delgI/gE/US9/US2 in suckling piglets

In investigating the safety of PRV GDFS-delgI/gE/US9/US2 as a potential live-attenuated vaccine, 5–7 days-old suckling piglets were inoculated intramuscularly with the recombinant virus. The DMEM group is the negative control group, whereas Bartha-K61 vaccine (10^5 $TCID_{50}$/Dose) is the positive control group. All suckling piglets were normal, and no clinical signs were observed throughout the experiment (Table 2). The rectal temperatures of all suckling piglets inoculated intramuscularly with the recombinant virus were below 40.0 °C. The results indicated that 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 was safe for suckling piglets.

3.3 Antibody production in piglets

PRV gE-specific antibodies were measured by competitive ELISA kit. All groups did not produce gE-specific antibodies before challenge (Figure 3A). After PRV GDFS wild-type strain challenge, the gE-specific antibodies were detected in all groups at 14 DPC. However, the gE-specific antibody levels in the 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine groups were significantly lower than those in the Bartha-K61 vaccine group.

PRV gB-specific antibodies were also measured by competitive ELISA kit. At 7 DPI, the gB-specific antibodies were detected in the vaccination groups (Figure 3B). The antibody levels of all vaccinated pigs peaked at 28 DPI. No significant differences in antibody level were detected between the 10^5 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 and 10^6 $TCID_{50}$ PRV

GDFS-delgI/gE/US9/US2 vaccine groups. However, the difference between the PRV GDFS-delgI/gE/US9/US2 and Bartha-K61 vaccine groups was significant. The gB-specific antibodies were not detectable in the DMEM group before challenge.

Serum samples were further evaluated for the ability to neutralize PRV by neutralizing test. o neutralization activity against the two different PRV strains was detected in the DMEM group before challenge. The 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine group induced high neutralizing titers against PRV GDFS strain (variant PRV strain) or PRV Ea strain (older PRV strain) (Figure 4). The neutralization titers peaked at 28 DPI in the vaccination groups. A significant difference was observed between the PRV GDFS-delgI/gE/US9/US2 and Bartha-K61 vaccine groups in neutralizing antibody titer. At 28 DPI, the mean neutralization titers induced by Bartha-K61 vaccine were higher against PRV Ea strain than that against PRV GDFS strain. Furthermore, the PRV GDFS-delgI/gE/US9/US2 vaccine showed enhanced cross-reactive neutralization antibody against variant PRV strain or older PRV strain.

3.4 Protection of immunized piglets against PRV challenge

The piglets of all groups were intranasally challenged with $10^{8.0}$ $TCID_{50}$/ml PRV GDFS strain at 28 DPI. The rectal temperature of all piglets was measured. In the DMEM group, all pigs displayed typical clinical signs (sneezes, breathlessness, loss of appetite, and dystaxia) with high fever (> 41 °C), and the pigs in the PBS group died at 7–12 DPC (Figure 5A). Three of the five pigs of the Bartha-K61 vaccine group showed fever at 3 and 7 DPC (ranging from 40.5 °C to 41.6 °C) and exhibited clinical signs, such as loss of appetite and sneezes. However, the pigs in the Bartha-K61 vaccine group survived after PRV GDFS strain challenge. The rectal temperatures of all piglets immunized with 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine were

below 40.0 °C after PRV GDFS challenge (Figure 5A). Moreover, no clinical signs were observed in pigs immunized with 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine.

The nasal swab samples were collected after challenge, and viral shedding was detected by viral isolation. As shown in Figure 5B, the titers of PRV shedding were detected in all groups. The titers of excretion of PRV in the DMEM group were higher than those in the vaccinated group. The titers of excretion of PRV in the Bartha-K61 vaccine group peaked at 4 DPC, and the average of PRV titer was $10^{3.65}$ $TCID_{50}$/ml. PRV shedding in the Bartha-K61 vaccine group was higher than those detected in the PRV GDFS-delgI/gE/US9/US2 vaccine groups. In addition, the difference between these 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine groups was not significant. The piglets in the 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine groups have already stopped shedding PRV at 6 DPC. However, PRV shedding in the Bartha-K61 vaccine group could still be detected at 8–10 DPC.

After the challenge, the piglets in the DMEM group showed poor growth and weight loss. The piglets in all vaccinated groups showed gain weight. The average weight gain in the Bartha-K61 vaccine group was significantly lower than those in the 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine groups.

3.5 Histopathological examination

Autopsy was performed to all dead and surviving piglets. No apparent gross lesions were found in the 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine groups. All dead piglets in the DMEM group showed severe brain hemorrhage and ulcer of the tonsil. No visible gross lesions in the tonsil were observed in the Bartha-K61 vaccine group, but all piglets showed slight hemorrhages in the brain. Histopathological analyses were further performed in the brain and tonsil. The histopathological lesions in the brain of the Bartha-K61 vaccine group were

perivascular lymphocyte infiltration and hemorrhage. The piglets in the DMEM group had perivascular lymphocyte infiltration, hemorrhage, and necrosis in the brain. Meanwhile, a large amount of inflamed cells were found in the tonsil. By contrast, no histopathological changes were observed in the 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine groups.

4 DISCUSSION

Since 2011, the mass outbreak of PR in China was primarily caused by virulence gene variation, which increased PRV virulence (Luo et al., 2014a). Various studies and clinical applications showed that the Bartha-K61vaccine only provided partial protection to the vaccinated pigs against the emerging PRV variants (Leng et al., 2013; Tong et al., 2015; Yu et al., 2017). PR has been considered as an economically important disease of the swine industry. At present, the vaccine is the economical and effective method to control PRV infection in China. On the basis of PRV strain variation, the gene-deleted inactivated vaccine or live-attenuated vaccine based on emerging PRV variants have been developed. For instance, JS-2012-△gI/gE, rPRVTJdelgE, rPRVXJ-delgI/gE-EGFP, PRV-HNX TK-/gE, rPRVTJ-delgE/gI/TK, vPRVHN1201TK-/gE-/gI-, rSMX△gI/gE△TK, and rZJ01△TK/gE/gI have been constructed (C. Wang et al., 2014; Hu et al., 2015; Z. Gu, Dong et al., 2015; Cong et al., 2016; Lei et al., 2016; Liang et al., 2016; Tong et al., 2016; Y. Yin et al., 2017; H. Yin et al., 2020). However, the immune protection indicates that the different doses of PRV vaccine or PRV challenge and different PRV strains can lead to different effects. In general, the protective efficacy of PRV vaccines showed that the live-attenuated vaccine was superior to inactivated vaccine.

In our earlier research, we have found that the CRISPR/Cas9 and Cre/Lox system can be used to develop a new PRV vaccine (Liang et al., 2016). Afterward, the CRISPR/Cas9 method has

been widely applied to edit PRV. The gene editing system dramatically increased the efficiency of the gene-deleted PRV strain. On this basis, Yan-Dong Tang et al. (2018) reported that CRISPR/Cas9 coupled with two sgRNAs could produce 100% knockout of PRV genes, which have provided an effective and powerful tool for PRV editing (Y. Dong et al., 2017; Tang et al., 2018).

The Bartha-k61 vaccine was developed after continued repeated passages in pig kidney cells, chicken eggs, and chicken embryo cells, and the 3489 bp fragments covering the complete gE and US9 genes and part of the gI and US2 genes have been deleted in Bartha-k61 genome by gene sequencing (B. Dong et al., 2014; Freuling et al., 2016). The Bartha-k61 vaccine is widely used to prevent and control PR worldwide, which has been proven safe and effective against PRV infection. However, since 2011, the PR mass outbreak in Barthak61-vaccinated pigs in swine farms of China demonstrated that the traditional Bartha-K61 vaccine could not provide complete protection against the emerging PRV variants in the field (Luo et al., 2014a, 2014b; Province et al., 2015; Tong et al., 2015; Article, 2016; Yu et al., 2017; J. Gu et al., 2018; Liu et al., 2018). In this study, we used two sgRNAs to remove nonessential genes between the two sgRNA target regions, where gI, gE, Us9, and Us2 are nonessential genes for PRV replication. We have developed a gI/gE/US9/US2-deleted attenuated vaccine on the basis of variant PRV strain (PRV GDFS), referring to the deletion of genes in Bartha-K61 genome by using the modified CRISPR/Cas9 technology. After the first round of plaque purification, these purified recombinant viruses were identified by PCR and gene sequencing. The results showed that recombinant viruses have produced 100% knockout. The recombinant viruses were purified by three rounds of plaque assay and were cultured after continued passages on PK-15 cells for 20 generations. The 20th

passaged PRV GDFS-delgI/gE/US9/US2 strain was used as vaccine virus seed, and its safety and efficacy were evaluated.

The safety of the vaccine is our first consideration in developing PRV live-attenuated vaccines. Suckling piglets are generally vulnerable to PRV infection that causes high mortality and even up to 100% death rate of infected piglets. Suckling piglets are generally preferred to be evaluated for the safety of PRV live-attenuated vaccines. In this study, 5–7 days-old suckling piglets inoculated with 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 were normal, and no clinical signs were observed throughout the experiment. The rectal temperatures of all suckling piglets inoculated intramuscularly with PRV GDFS-delgI/gE/US9/US2 vaccine were below 40.0 °C. These results indicated that PRV GDFS-delgI/gE/US9/US2 is safe for suckling piglets.

At present, conventional PRV live-attenuated vaccines have the ability of differential diagnosis, which allows differentiation of vaccinated from infected animals (DIVA). Therefore, DIVA strategies are performed by PRV gE-deleted vaccines combined with PRV gE-ELISA. In our study, the piglets immunized with 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 did not produce PRV gE-specific antibodies before challenge. This result indicated that PRV GDFS-delgI/gE/US9/US2 vaccine could serologically differentiate vaccinated animals from infected animals. On the contrary, all groups, except for the DMEM group, generated PRV gB-specific ELISA antibodies, and the PRV gB-antibody levels of the PRV GDFS-delgI/gE/US9/US2 vaccine group were higher than those of the Bartha-K61 vaccine group. Furthermore, the 10^5 $TCID_{50}$ or 10^6 $TCID_{50}$ PRV GDFS-delgI/gE/US9/US2 vaccine groups induced high neutralizing titers against PRV GDFS strain (variant PRV strain) or PRV Ea strain (older PRV strain). A strong association could be observed between the levels of neutralizing antibodies and protection against

PRV challenge. The PRV GDFS-delgI/gE/US9/US2 vaccine showed enhanced cross-reactive neutralization antibodies against variant PRV strain or older PRV strain.

In accordance with the manual of diagnostic tests and vaccines for terrestrial animals (Terrestrial Manual), the efficacy of PRV vaccines is evaluated by the four criteria after PRV challenge. Such criteria include the rectal temperature, weight loss, clinical signs, and mortality. In general, a high titer of the PRV virulent strain ($\geq 10^{7.5}$ $TCID_{50}$/ml) is recommended. In our study, a high-dose challenge with 10^{8} $TCID_{50}$/ml PRV GDFS virulent strain was performed by the intranasal route in all groups. After PRV challenge, all piglets immunized with PRV GDFS-delgI/gE/US9/US2 vaccine did not show any clinical signs, and the rectal temperature was normal. In addition, three of the five pigs of the Bartha-K61 vaccine group showed fever at 3 and 7 DPC and exhibited clinical signs such as loss of appetite and sneezes. The piglets in all vaccinated groups showed gain weight and no death. However, the average weight gain in the Bartha-K61 vaccine group was significantly lower than those of the PRV GDFS-delgI/gE/US9/US2 vaccine groups. Furthermore, the autopsy and histopathological analyses revealed that the piglets in the PRV GDFS-delgI/gE/US9/US2 vaccine groups did not show apparent gross and pathological lesions. All piglets in the Bartha-K61 vaccine group showed slight hemorrhages and pathological lesions in the brain. After PRV GDFS variant strain challenge, virus shedding was detected in all groups. PRV shedding in the Bartha-K61 vaccine group was higher than those detected in the PRV GDFS-delgI/gE/US9/US2 vaccine groups, and the excreted virus in the Bartha-K61 vaccine group lasted longer. The results of virus shedding were consistent with those of previous reports that PRV vaccines cannot completely prevent PRV infection. According to the criteria of OIE terrestrial manual, the results of the experiment

confirmed that PRV GDFS-delgI/gE/US9/US2 vaccine could provide full protection against emerging PRV variant strain in piglets compared with the commercial Bartha-K61 vaccine.

Therefore, a Bartha-K61-like vaccine based on emerging PRV variant was generated by the CRISPR/Cas9 method, which have deleted the gI, gE, US9, and US2 genes. The safety experiment has confirmed that PRV GDFS-delgI/gE/US9/US2 is safe for suckling piglets. The experiment on piglets challenged with emerging PRV variant strain showed that PRV GDFS-delgI/gE/US9/US2 vaccine confers complete protective immunity. In future studies, we will evaluate the efficacy and safety of PRV GDFS-delgI/gE/US9/US2 vaccine in pregnant sows.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interests with respect to the research, authorship or publication of this article.

ETHICS STATEMENT

All animal experiments in this study were approved by the Research Ethics Committee of College of Veterinary Medicine, Huazhong Agricultural University, Hubei, China.

DATA AVAILABILITY STATEMENT

PRV-GDFS was isolated from Guangdong Province of China and the gene sequence was submitted to the NCBI, accession number: MH521043.

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