**Evaluation of immunogenicity of gene-deleted vaccine and subunit vaccine constructed against the emerging variant pseudorabies virus**

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**Abstract:** Pseudorabies (PR) is an important infectious disease affecting pig farms worldwide. Since 2011, the emergence of variant strains of pseudorabies virus (PRV) had led to outbreak of PR in many vaccinated pig farms of China. Therefore, there is an urgent need to develop new vaccines against variant strains. In this study, based on the highly virulent PRV SD2017 mutant strain, the gene deleted strains SD2017ΔgE/gI and SD2017ΔgE/gI/TK-EGFP were constructed using homologous recombination technology. Using DC cell targeting peptide (DCpep) as an intramolecular adjuvant, and the outer membrane protein PorB of Neisseria meningitidis as a new protein adjuvant, PRV gB-DCpep and PorB protein containing gp67 protein secretion signal peptide were expressed using the baculovirus system. Rabbits that were intramuscularly vaccinated with PRV SD2017ΔgE/gI/TK live attenuated vaccine, PRV-gB+PorB subunit vaccine, and SD2017ΔgE/gI+PorB inactivated vaccine showed significantly higher PRV-specific antibodies, neutralizing antibodies, and intracellular cytokines. Through histopathological examination, it was found that no obvious histopathological damage was observed in the vaccinated rabbits. In addition, compared with the live attenuated vaccine, the PRV-gB+PorB subunit vaccine was effective against PRV infection. In general, the above three new vaccines were potential candidates for the development of PRV variant vaccines.

**Key words:** Pseudorabies virus; Recombinant virus vaccine; Subunit vaccine; Immune evaluation

## 1. Introduction

Pseudorabies virus (PRV), also known as porcine herpesvirus type I (SuHV1), belongs to the herpesvirus family and causes pseudorabies (Pseudorabies, PR), also named Aujeszky's disease (AD)(*1*). It can infect pigs (the only natural host), ruminants such as cattle and sheep, carnivores and rodents, with the mortality rate closing to 100% (*2*, *3*). PRV infects pigs at all stages, causing acute death of piglets, breeding problems in breeding pigs, and diseases of the nervous system and respiratory system of fattening pigs (*4*). It was reported that PRV has spread across multiple species and infected humans to cause endophthalmitis and encephalitis (*5*-*7*). The control of PR in China had been relatively stable untill the emergence of PRV variant strains in 2011. PR outbreaks occurred in pig farms even though vaccination with Bartha-K61 strain were administrated.(*8*, *9*). The Bartha-K61 strain vaccine showed limited protection against the variant strain, although this view was controversial (*10*, *11*). Since then, PRV

variants had spread in northern, eastern and southern China, caused serious damage to pig farms (*12-15*). Therefore, it was necessary to develop more effective vaccines against PRV variants.

PRV gB protein had been proven to induce neutralizing antibodies in animals, and gB protein could be used as a subunit vaccine target and diagnostic antigen. gE glycoprotein was the main virulence protein for PRV to invade the host nervous system(*16*). The virulence of gE deleted PRV was significantly attenuated, with reduced ability to invade the host's nervous system, including the trigeminal nerve and olfactory nerve endings (*17*). Therefore, the gE deleted PRV vaccine showed good safety(*18*). In addition, almost all wild PRV strains can express gE glycoprotein, and the gE gene was an important marker for the differential diagnosis of wild PRV infection(*19*).The gI and gE gene complexes and gC act together to mediate the release of the virus, affecting virus replication and virulence (*20-22*).The PRV non-structural protein thymidine kinase (TK) determined the virulence of the PRV virus, which was related to virus replication and latent infection, and played an important role in the virus proliferation of the host central nervous system(*18*).

In previous studies, we isolated and identified a highly virulent PRV variant SD2017 strain. Sequence analysis showed that it is closely related to PRV variants that have recently emerged in China(*10*). Based on the PRV variant SD2017, we constructed an inactivated ΔgE/gI vaccine and a live attenuated vaccine lacking gE/gI/TK, respectively. And based on gB and PorB proteins, a new subunit vaccine PRV gB-DCpep was prepared. The pathogenicity, immunogenicity and clinical protection of these PRV vaccines were evaluated in rabbits. The results showed that the PRV SD2017ΔgE/gI/TK live attenuated vaccine, PRV-gB+PorB subunit vaccine and SD2017ΔgE/gI+PorB inactivated vaccine can provide effective protection against the highly virulent PRV SD2017 strain. Among them, the PRV SD2017ΔgE/gI/TK live attenuated vaccine had the best immune effect.

## 2. Materials and Methods

### 2.1. Virus and Cell Lines

The wild-type PRV virulent SD2017 strain was isolated in 2017 from a pig farm in Shandong, China. Based on PRV SD2017 gE gene (MW535259) sequencing and genetic evolution analysis, it was identified as a PRV variant strain circulating in China. PRV Bartha-K/61 strain was a classic vaccine strain for Pseudorabies control and eradication. The PK-15

cell line was used to culture PRV. PK15 cells were cultured in Eagle Medium (DMEM, Gibco, USA) modified with 10% Fetal Bovine Serum (FBS, Gibco, USA) at 37°C in 5% $CO_2$ a humidified incubator. Sf9 cells were used to propagate recombinant baculovirus. They were cultured in Grace insect medium (Gibco, USA) with 2% FBS at 27°C.

### 2.2. Primers and plasmids

Homologous recombination technology was used to construct the gE/gI and TK gene deleted strains of PRV SD2017. The recombinant virus construction strategy (Fig. 1) was based on the pBluescript II KS(+) cloned plasmid as the backbone. The homology arms of gE/gI gene and TK gene were inserted into pBluescript II KS(+) respectively (Table 1). The complete EGFP gene was inserted between the two homology arms for screening of recombinant viruses. Recombinant plasmids pBKS-gIL/gER and pBKS-gIL/gER-EGFP, pBKS-TKL/R and pBKS-TKL/R-EGFP were constructed respectively.

### 2.3. DNA extraction and transfection

A commercially available kit (OMEGA, USA) was used to prepare the plasmid. PRV SD-2017 strain was cultivated in PK-15 cells, and then purified by 20-60% sucrose density gradient centrifugation (130,000g, 4°C, 2h). Viral DNA was purified from infected cells or purified virus by DNAiso Reagent (Takara, China). The plasmid and viral DNA were transfected using Calcium Phosphate Cell Transfection Kit (Thermo scientific, USA), following the manufacturer's instructions.

### 2.4. Generation of PRV mutants

Homologous recombination method was used to generate virus mutants(*23*, *24*). Follow the steps in the Thermo Fisher calcium phosphate transfection kit instructions, the PRV SD2017 genomic DNA (gDNA) and pBKS-gIL/gER-EGFP were co-transfected into PK-15 cells (5×105 cells/well). When the green fluorescent plaque was identified, the plaque was marked under a fluorescent microscope, and recombinant virus was obtained through plaque purification. PCR detection and sequencing analysis of gE/gI gene deletion and gB gene existence were performed on the recombinant virus. After multiple rounds of plaque purification, a reconstructed virus SD2017ΔgE/gI-EGFP lacking TK gene and expressing green fluorescence was obtained.

As described above, by co-transfecting SD2017ΔgE/gI-EGFP DNA and pBKS-gIL/gER

recombinant plasmid into PK-15 cells, the non-fluorescent virus plaques were purified to obtain the recombinant virus SD2017ΔgE/gI without EGFP marker gene. The recombinant plasmid pBKS-TKL/R-EGFP and SD2017ΔgE/gI genomic DNA were co-transfected into PK-15 cells. After multiple rounds of virus plaque purification, the recombinant virus SD2017ΔgE/gI-EGFP expressing green fluorescence was obtained.

### 2.5. PRV growth kinetics and plaque size determination

The growth kinetics of PRV in PK-15 cells was studied as previously described (Smith and Enquist, 1999). PK-15 cells were grown to 80% confluence in a 24-well plate and infected with PRV SD-2017, SD-2017ΔgE/gI, SD-2017ΔgE/gI-EGFP, Bartha-K61 virus at the same multiplicity of infection (MOI=1.0) . The virus was allowed to adsorb at 37°C for 1 h. The virus was removed, cells were washed twice with PBS, and then replaced with fresh DMEM medium. The culture supernatants were tested at 4, 8, 12, 24, 36, 48, 60 and 72 h post-infection (hpi)by inoculating the cell monolayers at an MOI of 1. Virus titers were expressed as 50% tissue culture infection dose ($TCID_{50}$) and were recorded as $TCID_{50}$/ml. All experiments were repeated at least 3 times, the average titer was calculated, and the growth kinetic curve of each tested virus was drawn.

Five hundred $TCID_{50}$ of PRV was inoculated on PK-15 cells inoculated in a six-well plate for 48 hours, 1% agarose medium was added and the plaque size was measured 1 hour later. For each virus, 100 plaques were randomly selected, and their size was determined by ImageJ software (NIH).2.7.

### 2.6. Expression of PRV gB and Meningococcus PorB fusion proteins in recombinant baculovirus

Plasmids expressing the gB-DCpep fusion protein of PRV SD2017 strain and the PorB protein of Neisseria meningitidis were constructed respectively. PCR-based gene assembly methods were used to generate fusion proteins (Fig. 1A and B). The fusion construct was verified by DNA sequencing. The purified fusion construct gB-DCpep and PorB were digested with pFastBac 1 to construct a baculovirus transfer vector, which was named pFastBac1-gB-DCpep and pFastBac1-PorB. Then according to the manufacturer's instructions, the Bac-to-Bac system was used to generate recombinant baculovirus Ac-gB-DCpep and Ac-PorB (Fig.1C).

### 2.7. Detection and purification of PRV gB-DCpep and MC PorB

Sf9 cells in logarithmic growth phase were infected with recombinant baculovirus (Ac-gB-DCpep and Ac-PorB) at a multiplicity of infection (MOI) of 1.0 and cultured at 27°C for 60 h. Then, proteins were extracted from the culture supernatant and analyzed by Western blot analysis. Samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked overnight at 4°C in Tris buffer and Tween 20 (TBS-T) containing 5% non-fat milk , and anti-His monoclonal antibody (1:5000, Proteintech, USA) Incubate for 2h at room temperature. HRP-labeled goat anti-mouse antibody (Jackson, USA) was used as the secondary antibody, and the membrane was incubated for 1 h at room temperature. After washing three times with TBS-T, the signal was visualized using an ECL chemiluminescence system.

To purify PRV gB-DCpep and PorB proteins, Sf9 cells were infected with recombinant baculovirus Ac-gB-DCpep and Ac-PorB at a MOI of 0.1 respectively. After infection for 120 hours, the culture supernatant was centrifuged at 12000 rpm for 30 minutes, and filtered with a 0.22 μm filter to remove residual cell debris. PRV gB-DCpep and PorB proteins were purified by AKTA protein purification system and passed through a nickel affinity chromatography column (GE, USA) according to the manufacturer's instructions. Then, the purified PRV gB-DCpep and PorB proteins were confirmed by 12% SDS-PAGE, and the BCA protein assay kit (Vazyme, China) was used for quantitative analysis.

**2.8. Determination of $LD_{50}$**

Healthy young rabbits weighing 2~2.5 kg were randomly divided into 11 groups (n=5), and were innoculated with PRV SD-2017 ($10^2$~$10^5$ $TCID_{50}$/ml) and SD-2017ΔgE/gI ($10^3$~$10^5$ $TCID_{50}$/ml), SD-2017ΔgE/gI/TK ($10^4$~$10^6$ $TCID_{50}$/ml), to determine $LD_{50}$ and evaluate the virulence of each PRV strain. Rabbit was inoculated intranasally with 0.5 mL of PRV virus, and 0.5 mL of DMEM in the control group. The clinical symptoms and deaths of each group were observed daily within 14 days after inoculation, and the mental state, feeding, itching, body temperature and other clinical symptoms of each group were recorded. The Karber method was used to calculate the $LD_{50}$ of each strain. All animal experiments in this study were carried out in standardized animal facility of Shandong Huahong Biological Engineering Co., Ltd., and the experimental protocols have been approved by the Laboratory Animal Welfare

and Ethics Management Committee.

### 2.9. Immunization and challenge of Rabbit

Healthy young rabbits weighing 2-2.5kg were randomly divided into six groups (n = 10). The rabbits in each group were immunized by intramuscular injection of the vaccine according to Table 2. DMEM as blank control (Group G). Three weeks after vaccination, the same dose was used for booster immunization. Six weeks after the final immunization, the immunized rabbits were infected through the nose with the virulent PRV SD 2017 strain (100 $LD_{50}$). The attacked rabbits were checked every day to quantify the number of dead animals and calculate the survival status.

### 2.10. Evaluation of humoral and cellular immune response post immunization

The PRV-specific antibodies (IgG, IgG1, IgG2a) in the serum were determined by indirect ELISA . The purified gB protein was coated to a 96-well microtiter plate at 0.5 μg per well, in the coating buffer (pH 9.5) at 4°C overnight. 1% bovine serum albumin was used for blocking at 37°C for 1 h. Then, serially diluted serum samples were added and incubated at 37°C for 1 hour. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000), IgG1 (1:5000) and IgG2a (1:5000) were added to measure PRV-specific antibodies (IgG, IgG1 and IgG2a) respectively. The antibody endpoint titer was determined based on the highest dilution which gave an $OD_{450}$ twice that of the naïve group without dilution.

Serum neutralization test was used to detect PRV antibodies. The serum samples were diluted with DMEM and added to 96-well cell culture plates. 50 μL of 200 $TCID_{50}$ PRV SD2017 strain virus were added to each well. After incubating at 37°C for 1 hour, 50μL PK-15 cell suspension was added to each well. After 3 days, the PRV-specific neutralizing antibody titer was calculated and expressed as the reciprocal of the highest dilution inhibiting PK-15 cell infection.

Lymphocyte proliferation were analyzed using MTS assay kit. Peripheral blood mononuclear cells (PBMC) were prepared by peripheral blood lymphocyte separation solution. 100μl of PBMCs in of RPMI 1640 cell culture medium containing 10% FBS were added to a 96-well plate at denstity of $4\times10^6$ cells/ml, Concanavalin A (PHA) (10μg/mL) was added, a negative control group was set without PHA. After incubation in a 37°C 5% CO2 incubator for 72 hours, 20 μL of MTS (5 mg/mL) were added to each well. After 4 hours of incubation at

37°C, the stimulation index (SI) was calculated according to the following formula: SI = (OD value of the immune group-OD value of the control group)/(OD value of the negative control group-OD value of the control group).

In order to fully understand the cellular immune response induced by immunization Th1 cytokine (IFN-γ) and Th2 (IL-6) cytokine level in the serum were evaluated by ELISA kit (Jingmei, China) according to the instruction of manufacture post immunization.

### 2.11. Necropsy and pathological examination

Rabbits that died after the challenge were subjected to necropsy immediately. The pathological changes of the brain, liver, lung, kidney and other organs were recorded. After the challenge, the surviving rabbits were euthanized by injecting an excessive amount of intraperitoneal pentobarbital sodium. The brain, liver, lung, and kidney organs were taken, fixed in 10% formalin, and embedded in paraffin. The buried tissue samples were cut into 4μm-thick sections, stained with HE, and pathological changes were observed under an optical microscope.

### 2.12. Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Student's t-test was used to compare means between two groups. Oneway ANOVA followed by Tukey's test was used for multiple comparisons. Statistical significance was set at $p<0.05$, and extremely significant was set at $p<0.01$.

## 3. Results

### 3.1. Characterization and growth characteristics of PRV virulence gene deleted strains

In this study, pBKS-gIL/gER-EGFP and PRV genome were co-transfected in PK-15 cells at a ratio of 3:7 for 48 hours. After observing a large number of green fluorescent plaques, the cells showed obvious CPE. A total of 6 rounds of virus plaque purification were performed to obtain SD-2017ΔgE/gI-EGFP with gE/gI knockout and EGFP expression (Fig.3A). SD-2017ΔgE/gI-EGFP DNA and pBKS-gIL/gER were co-transfected into PK-15 cells. After 10 rounds of screening and purification of virus plaques without green fluorescence, gE/gI knockout and EGFP-free virus plaques were obtained SD-2017ΔgE/gI, and nucleotide sequencing confirmed that the gE/gI gene has been deleted. According to the same method as above, we had screened and purified SD-2017ΔgE/gI/TK-EGFP which lacking TK/gE/gI and

expressing EGFP (Fig.3A). PCR identification showed that the gB gene was detected in the genome of the 2017ΔgE/gI/TK-EGFP, but the TK gene was not detected, which was consistent with the nucleotide sequencing results. At the same time, it was found that the growth kinetics of SD-2017ΔgE/gI/TK-EGFP strain and SD-2017ΔgE/gI strain were slightly slower than that of the parental strain SD-2017 and the traditional vaccine strain Bartha K61 (Fig.3B). The titers of the two recombinant viruses were $10^{8.0}TCID_{50}$/mL after culturing for 60h, but the plaque size of the virus was smaller than that formed by SD-2017 strain and Bartha K61 strain (Fig.3C), indicating that genetic manipulation influeced the PRV proliferation.

**3.2. Expression and purification of recombinant protein PRV gB-DCpep and PorB**

In order to confirm the expression of PRV gB-DCpep and PorB protein, the protein from P3 generation recombinant baculovirus infected sf9 cells was purified by Ni-NTA chromatography, and western blot analysis was performed. SDS-PAGE results showed that the supernatant of sf9 cells infected with recombinant baculovirus Ac-gB-DCpep had bands at 82, 47 and 35 kDa (Fig. 4A). The anti-His monoclonal antibody detected bands at 82 and 35 kDa respectively (Fig. 4A). Among them, 82kDa was the full-length PRV gB-DCpep protein, 47 kDa was the N-terminal degradation fragment of PRV gB-DCpep protein, and 35 kDa was the C-terminal degradation fragment of PRV gB-DCpep protein. In sf9 cells infected with recombinant baculovirus Ac-PorB, a band of anti-His monoclonal antibody was detected at 36 kDa in the whole cell sample (Fig. 4B), but no band was detected in the supernatant. In contrast, no specific protein was produced in normal cells (negative control). In summary, these results had shown that PRV gB-DCpep and PorB proteins were confirmed and purified.

**3.2. Virulence of PRV Gene Deleted Strains**

The Karber method was used to calculate the $LD_{50}$ of each strain of PRV in rabbits (Table 3). The results have shown that the PRV SD-2017 strain has certain virulence after gE/gI gene deletion ($LD_{50}$ was $7.94\times10^4$ $TCID_{50}$). After gE/gI/TK gene deletion, the virulence of the virus was be reduced to $LD_{50}>1.0\times10^6TCID_{50}$. Rabbits that survived the observation period were euthanized. The brains, livers, and kidneys were fixed and made into tissue sections, then stained with HE to observe the pathological changes. SD-2017ΔgE/gI group showed mild perivascular space was widened in brain tissue, some liver cells were swollen and necrotic, and the renal tubular epithelial cells were swollen. There were no obvious lesions in the SD-

2017ΔgE/gI/TK and DMEM control groups (Fig.5).

### 3.4. Humoral immune response following immunization

In order to determine the humoral immune response caused by each group of vaccination, IgG and IgG isotypes (IgG1 and IgG2a) in serum were evaluated by ELISA. Except for the control group, all groups had produced PRV-specific IgG antibodies at 14, 28, and 42 dpi. and the live vaccine immune group produced higher antibodies at 14 dpi. In addition, by 42dpi, the IgG antibody titer of rabbits immunized with PRV-gB+PorB subunit vaccine and SD2017ΔgE/gI/TK(+) live attenuated vaccine were significantly higher than that of other groups (Fig. 6A). At 28dpi and 42dpi, the measurement of IgG isotype titers (IgG1 and IgG2a) had shown the results (Fig.6B, Fig. 6C), using PRV-gB+PorB, SD2017ΔgE/gI/TK(+), Bartha K61 (+) The immunized rabbits induced high expression levels of IgG1, which was an indicator of Th2 immune response. Obviously, compared with other immunization groups, the SD2017ΔgE/gI/TK(+) immunization group produced a significantly higher amount of IgG2a. The results had shown that the above-mentioned vaccines can effectively promote humoral immune responses.

Neutralizing antibodies were essential for evaluating the immune effect of vaccines. Therefore, this study further analyzed the levels of PRV neutralizing antibodies in each group (Fig.6D). No neutralizing antibodies against PRV SD-2017 strain were detected in the control group. In contrast, all vaccinated groups showed PRV SD-2017 strain-specific neutralizing antibody responses at 14, 28, and 42 dpi. The antibody titer of PRV-gB+PorB and SD2017ΔgE/gI/TK(+) group was significantly higher than other groups at 28 and 42dpi. In addition, compared with other groups, the attenuated live vaccine SD2017ΔgE/gI/TK(+) significantly increased the titer of neutralizing antibodies at 14dpi. In summary, the results had shown that the recombinant protein PRV-gB+PorB and the attenuated live vaccine SD2017ΔgE/gI/TK(+) have good immunogenicity and effectively increase the titer of the antibody.

### 3.5. Cellular immune response induced by PRV immunization

In order to further study the cellular immune response, the proliferation index (SI) of peripheral blood lymphocytes of rabbits were measured on the 42nd day after immunization(Fig.7A). At 42 dpi, the SI difference between immunized group and DMEM

group was significant. In addition, the PRV-gB+PorB and SD2017ΔgE/gI/TK(+) groups showed significantly higher peripheral blood lymphocyte stimulation index (SI) than the other groups. IL-6 and IFN-γ cytokine levels, which were related to Th2 bias and Th1 cell response, respectively were measured by ELISA kits in the peripheral blood The results showed that at 42dpi, compared with the DMEM group, the secretion of IL-6 and IFN-γ in all vaccine groups increased (Fig.7B, Fig.7C). Moreover, the IL-6 and IFN-γ concentrations of PRV-gB+PorB, SD2017ΔgE/gI/(-)+PorB, SD2017ΔgE/gI/TK(+), Bartha K61(+) group were significantly higher than PRV-gB or SD2017ΔgE /gI/(-)+206 groups. These data showed that the combined immunization of gB-DCpep and PorB protein significantly enhanced the cellular immune response.

### 3.6. Immune protection of vaccines against PRV variant strains

In order to evaluate the immune protective effect of the vaccinated rabbits against the virulent PRV variant strains, rabbits were intranasally inoculated with 100 $LD_{50}$ virulent PRV SD-2017 strains. During the 14-days monitoring period, the survival rates of rabbits (n=10/group) were illustrated in Fig. 8. the rabbits immunized with the attenuated live vaccine SD2017ΔgE/gI/TK(+) showed a 100% protection rate, and rabbits vaccinated with the recombinant proteins PRV-gB+PorB and SD2017ΔgE/gI(-)+PorB showed 90% protection rate. Rabbits immunized with recombinant protein PRV-gB and SD2017ΔgE/gI(-)+206 showed a protection rate of 80%, while those immunized with Bartha K61(+) had a protection rate of 50%. The results suggested that the attenuated live vaccine, inactivated vaccine and recombinant protein subunit vaccine immunization group prepared based on the PRV variant strain SD-2017 all have a protection rate of more than 80% against the virulent PRV infection. In addition, the attenuated live vaccine SD2017ΔgE/gI/TK(+) had the best protective effect (100% survival rate).

Additionally, as shown Fig. 9, the histopathology examination results indicated that in rabbits immunized with DMEM and live attenuated vaccine Bartha K61(+), there was obvious perivascular space and accompanied by neurophagy in the brains, obvious congestion and edema in the lungs, congestion in the liver, swelling and necrosis of some liver cells, hemorrhage in the kidney, and necrosis and shedding of some renal tubular epithelial cells. Whereas, no obvious histopathological lesions or slight pathological damage were observed in

rabbits immunized with SD2017ΔgE/gI/TK(+) live attenuated vaccine, PRV-gB+PorB subunit vaccine and SD2017ΔgE/gI(-)+PorB inactivated vaccine. In conclusion, these data demonstrated that the three new genetic engineering vaccines constructed above were efficient for PRV infection in rabbits.

## 4. Discussion

Vaccination is one of the most effective methods to prevent and control PR. The live PRV vaccine with gE gene deletion has been widely used in China for long-term control of the disease(*24*). At present, the most widely used vaccine strain Bartha-K61 has a 3489bp nucleotide deletion at positions 122560~126049, including part of the gI gene, the full length of gE and Us9 genes, and part of the Us2 gene(*24*, *25*). However, since 2011, PR outbreaks by new highly pathogenic PRV variants with unique molecular characteristics occured in most areas of China despite that vacccination were administrated in pig farms At present, there have been reports of gE/gI gene deletion or TK/gE/gI gene deletion vaccines constructed against variant strains. Compared with traditional Bartha-K61 vaccines, those vaccines can provide higher protection against PRV variants(*26*).

In our previous study, a highly virulent PRV variant SD-2017 were isolated and identified. Sequence analysis showed that the SD-2017 strain is closely related to other PRV variants that have recently appeared in China, and these variants have been isolated by different research teams. In order to control the infection of highly pathogenic PRV variant strains, we have developed a gI/gE double-gene deleted inactivated vaccine and a gI/gE/TK triple-gene deleted live vaccine based on the PRV SD-2017 variant strain. The results of rabbit vaccination and protection test showed that compared with Bartha-K61 vaccination, the above-mentioned vaccine can protect rabbits from the lethal infection of SD-2017 strain by 80%~100%. In this study, the SD-2017ΔgE/gI/TK-deficient live vaccine has provided complete protection against the challenge of the SD-2017 strain and can be developed as an ideal vaccine candidate. In addition, the combined immunization with the inactivated antigen of PRV SD-2017ΔgI/gE strain and PorB protein is better than the combined immunization with 206 adjuvant, and the protection rate against SD-2017 strain is 90%, suggesting that PorB protein had a good immunostimulatory effect and can be applied to the preparation of PRV SD-2017ΔgI/gE

inactivated vaccine. The Bartha-K61 vaccine had only a 50% protection rate against SD-2017 strain, the level of neutralizing antibodies was low, and the rabbits still have obvious pathological damage after challenge,indicating that the Bartha-K61 vaccine had limited protection against SD-2017 strain infection.

PRV glycoproteins gB, gC and gD are the main antigens that induce PRV humoral and cellular immune responses(*27*). Recombinant DNA vaccines of gC and gD can induce both humoral and cellular immune responses against PRV infection (*28*, *29*). Previous studies have evaluated the induction of cellular immunity by preparing gB, gC, and gD recombinant heterologous vector vaccines in mice and pigs (*29-31*). In addition, the high level of cell-mediated immunity has an effect on preventing virus shedding(*32*). In this study, we focused on the construction of PRV glycoprotein gB subunit vaccine. By using DCpep as a molecular adjuvant and PorB as a new protein adjuvant, recombinant baculovirus Ac-gB-DCpep and Ac-PorB expressing PRV SD-2017gB-DCpep fusion protein and MC PorB protein were successfully constructed. PRV glycoprotein was effectively expressed in the culture supernatant of sf9 cells, and PorB protein was expressed in the cells.

Most genetically engineered subunit vaccine are composed of two main components: antigen and adjuvant. The antigen is a small molecule against which the vaccine induces an immune response and provides protection through the immune stimulating ability of the adjuvant. PorB protein can induce more follicular dendritic cells (FDC) and more antigen accumulation on FDC, and has the ability to increase FDC-antigen interaction, which is essential for inducing B cell affinity maturation(*33*). Previous studies have verified the immunogenicity of recombinant PorB eggs against porcine circovirus type 2 and Mycoplasma hyopneumoniae genetically engineered vaccines, which can enhance the humoral and cellular response levels of mice induced by the two genetically engineered vaccines in a short period of time(*34*). In this study, a subunit vaccine prepared by mixing PorB protein and gB-DCpep fusion protein was used to immunize rabbits. The results showed that gB-PorB protein can induce humoral and cellular immune response, and can increase PRV-specific IgG and IgG isotypes (IgG1 and IgG2a) level, and significantly increased the titer of neutralizing antibody at 28 and 42 dpi. The levels of Th1 and Th2 cytokines increased after antigen stimulation. The survival rate of immunized rabbits against SD-2017 strain is 90%. No obvious histopathological

damage was observed in the rabbits after challenge, while the immunized Bartha live vaccine group showed obvious histopathological damage. Although the protection rate of gB-PorB protein subunit vaccine is lower than that of PRV SD2017ΔgE/gI/TK three-gene deletion live vaccine (100%), compared with the risk of individual safety of PRV live vaccine(*35*), the gB subunit vaccine still has important development value.

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**Table.1 Primers used for PRV gE/gI gene and TK gene knockout**

**Table.2 Groups of PRV vaccine in rabbit immunity test**

Note, (+) meant live vaccine. (-) meant inactivated vaccine. 206, ISA 206 adjuvant.

**Table.3 Result of rabbit $LD_{50}$ determination test**

**Fig. 1. Strategy for the construction of the SD2017 ΔgE/gI strain and ΔgE/gI/TK-EGFP strain**

(A) Schemic representation of SD2017 genome. (B)Schemic representation of SD2017ΔgE/gI genome. (C) Sites of gene replacements and the genome organization of the SD2017ΔgE/gI/TK/-EGFP strain.

**Fig.2 . Schematic representation of the recombinant protein constructs.**

(A) Schematic representation of the genetic fusion of GP67 + SD-2017 gB + Linker + DCpep-His. (B) Schematic representation of the genetic fusion of GP67 + MC PorB-His

**Fig. 3. Identification of the genetically deleted PRV strains.**

(A) Cytopathic effect and EGFP expression of SD-2017ΔgE/gI-EGFP and SD-2017ΔgE/gI/TK-EGFP infected cells. (B) Viral growth curves of the SD-2017, SD-2017ΔgE/gI, SD-2017ΔgE/gI/TK, and Bartha K61 strains. (C) Plaque size of the indicated viruses.

**Fig. 4. Detection and purification of PRV gB-DCpep and PorB proteins.**

(A) Detection and purification of PRV gB-DCpep protein. (B) Detection and purification of PRV gB-PorB protein. Lane 1, The purified gB-DCpep protein was examined through SDS-PAGE. Lane 2, gB-DCpep protein from culture supernatant of sf9 cells was detected using western blot analysis. Lane 3 and Lane 4, negative control: culture supernatant of sf9 cells infected without recombinant baculoviruses. Lane 5, PorB protein from culture the whole of sf9 cells was detected using western blot analysis.

**Fig. 5. Pathogenicity of the genetically deleted PRV strains.**

(A) The perivascular space was widened. (B) Liver cells were swollen and necrotic. (C) The renal tubular epithelial cells were swollen, and the lumen was reduced or atresia. (D, E, F, G, H, I) No obvious pathological changes were seen.

**Fig. 6. Antibody titer in immunized rabbits.**

(A) The gB-specific IgG titer from serum samples was determined by indirect ELISA at 14, 28 and 42 dpi. (B) PRV-specific IgG1 and (C) IgG2a titers were detected at 28 and 42 dpi by ELISA. (D) Neutralizing antibody titers against PRV SD-2017 strain. The neutralizing ability

of antisera generated against PRV SD-2017 strain were calculated and expressed as the log2 of the reciprocal of the highest serum dilution when PK-15 cell infection was inhibited. n = 10. *, $p < 0.05$. **, $p < 0.01$. #, $p < 0.05$. ##, $p < 0.01$. Note, (+) meant live vaccine. (-) meant inactivated vaccine. 206, ISA 206 adjuvant.

**Fig. 7. Cellular immune response analysis of immunized rabbits.**

(A) Lymphocyte proliferation test at 42 dpi. (B) The cytokine IFN-γ and (C) IL-6 levels in the supernatant of stimulated T cells were determined by cytokine ELISA at 42 dpi. n = 10. *, $p < 0.05$. **, $p < 0.01$. #, $p < 0.05$. ##, $p < 0.01$. Note, (+) meant live vaccine. (-) meant inactivated vaccine. 206, ISA 206 adjuvant.

**Fig. 8. Protective immunity to rabbits intranasally infected with virulent PRV SD-2017 strain.**

Three weeks after the second immunization, rabbits in seven groups were intranasally challenged with 100 $LD_{50}$ of PRV SD-2017 strain. Percentage survival on the indicated days was calculated as: survival rate (%) = (numbers of mice surviving/numbers of total rabbits)×100%. Note, (+) meant live vaccine. (-) meant inactivated vaccine. 206, ISA 206 adjuvant.

**Fig. 9. Histopathological observation of rabbits in different vaccination groups after being challenged (HE)**

In immunized rabbits at 14 d post-challenge, HE staining was used to detect the pathological lesions of brains, Lungs, Livers and Kidneys tissues. Scale bar, 100 μm or 200 μm. The black arrow indicated the widening of the perivascular space. The red arrow indicated congestion in the lungs. The purple arrow indicated lung edema. The yellow arrow indicated liver congestion, and some stem cells were swollen and necrotic. The green arrow indicated renal hemorrhage, congestion, and swelling of some glomeruli. A. PRV-gB, B. PRV-gB+PorB, C. SD2017ΔgE/gI(-)+206, D. SD2017ΔgE/gI(-)+PorB, E. SD2017ΔgE/gI/TK(+), F. Bartha K61(+), G. DMEM. HE, hematoxylin and eosin staining. 206, ISA 206 adjuvant.