Pseudorabies virus (PRV) strain with defects in gE, gC, and TK genes protects piglets against an emerging PRV variant

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ABSTRACT. The prevalence of an emerging variant of the pseudorabies virus (PRV) has been causing serious losses to farmers in China. Moreover, the commercially available PRV vaccine often fails to provide thorough protection. Therefore, in this study, we generated a PRV-ΔgC\gEΔTK strain with defects in gC, gE, and TK of PRV. Compared to the parental PRV strain and the single gene deletion strains (PRV-ΔgC, PRV-ΔgE, and PRV-ΔTK), PRV-ΔgC\gEΔTK grew slowly, and exhibited fewer and smaller plaques on swine testis (ST) cells. Furthermore, animal experiment results showed that mice that were immunized intramuscularly with PRV-ΔgC\gEΔTK, survived throughout the experiment with no observed clinical symptoms, and were completely protected against PRV challenge. Additionally, deletion of the gC, gE, and TK genes significantly alleviated viral damage in the brain. Furthermore, one-day-old weaned piglets immunized intramuscularly with PRV-ΔgC\gEΔTK elicited higher levels of gB antibodies against both the emerging PRV variant and the parental PRV, exhibited full protection against challenge with both variants, and showed neutralization capacity against PRV. These data suggest that PRV-ΔgC\gEΔTK is a promising vaccine candidate for the control of pseudorabies.

KEY WORDS: gC/gE/TK deletion, PRV-ΔgC\gEΔTK, pseudorabies virus, vaccine

Pseudorabies virus (PRV) is a member of the genus Varicellovirus, belonging to the PRV family Alphaherpesvirinae, which can infect numerous livestock and wild animals, with swine being the natural viral reservoir [3, 24]. Infected pigs manifest various clinical symptoms, including nervous system disorders and high mortality in young piglets, respiratory disorders and growth retardation in mature pigs, and reproductive failure in sows. In the last decade, pseudorabies (PR) has been well-controlled in China, owing to the Bartha-K61 vaccine. However, since late 2011, PR has shown occurrences even in Bartha-K61-vaccinated pigs, particularly in those that were intensively inoculated. Recent evidence has further indicated that the Bartha-K61 live vaccine was incapable of providing sufficient protection against novel variants [2, 21, 22, 30]. Thus, Chinese farms urgently require the development of a novel vaccine to control the emerging PRV variant. At present, various vaccines have been reported, such as inactivated PRV (Fujian strain) and HB (Hubei strain) vaccines, as well as live attenuated vaccines of the HB-98 strain with a TK/gC double gene deletion, apart from SA215 strain (PRV) with a gE/gI/TK gene deletion, and HB2000 strain with a TK/gG/gE 3 gene deletion [2, 6, 12, 28]. However, these vaccines have been shown to be poorly effective at protecting pigs, according to recent clinical data, which revealed an average positive PRV rate of 8.27% between 2012 and 2017, indicating that PRV-vaccines currently used in China still show a high risk of PR prevalence [8, 10].

The PRV strain of Fa is the earliest isolated typical strain that caused PR prevalence in China, resulting in major economic losses for the pig industry [9]. Phylogenetic analysis has suggested that most of the newly isolated PRV strains in China in recent years originated from evolution of the PRV strain, some of which are very closely related, such as HuB-China-2016, GD-QY-2010, and GD-JM-2015 [10, 26]. Therefore, the PRV strain is suitable as a template for vaccine development. Previous reports have indicated that a gene-deficient strain derived from the PRV strain, SA215 (ΔgE/gI-/ΔTK-), has high immunogenicity and long-term immunity, and was developed for use as a commercial live-attenuated vaccine [15, 17, 30].

Preliminary studies have shown that the transneuronal transfer function of gE and gI may be mediated by the formation of complexes; the absence of gE also affects gI function *in vivo*, thus making the deletion of gI unnecessary during vaccine

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development [4, 7, 16, 31]. However, the gC protein is a major virulent protein of PRV, involved in not only guiding the adsorption process between the virus and target cells, but also in nervous system invasion and release [18]. Previous sequence alignment results have identified many amino acid changes within gC proteins of the PRV circulating in China after the outbreak compared to those from the viruses prevalent in China before the outbreak. This indicates that these changes may also be associated with changes in the virulence of the virus [8]. Therefore, the PRV-vaccine with gC and TK deletion could potentially be developed into an effective commercial live-attenuated vaccine [8, 23].

In this study, we first constructed a live-attenuated PRV strain carrying deletions for gC, gE, and TK using CRISPR technology, *in vitro*. The safety and immunological efficacy of this strain, PRV-ΔgC\gEΔTK, were then evaluated. PRV-ΔgC\gEΔTK was also compared with commercial vaccines generated from SA215 strains.

MATERIALS AND METHODS

Plasmid construction and virus

The stably expressing CRIPSR/Cas9 plasmid system used in this study was constructed by introducing sgRNA (targeting various genes and PRV genes) into a lenti-CRISPR V2 plasmid (Addgene, Watertown, MA, USA) [4, 5, 13, 19, 20, 25]. All target sites and gRNA sequences used in this study are shown in Table 1. The lenti-CRISPR V2 vector was cleaved with endonuclease Esp3I (*BsmBI*) (Thermo Fisher Scientific, Waltham, MA, USA). The sgRNA was ligated into the vector using T4 DNA ligase (Takara China, Dalian, China). The procedures for producing stably expressing CRIPSR/Cas9 plasmid systems were followed. PRV-Fa and PRV variants (201715 strains) were isolated in 2017 from an infected pig farm in China, and consequently propagated in Vero cells.

Cell culture and transfection

Both 293T and swine testis (ST) cells were cultured in Dulbecco's modified Eagle medium (DMEM) plus 10% FBS (GIBCO, Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2. Plasmids were transfected into cells using Lipofectamine 3,000 (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, $2 \times 10^{5.0}$ cells were cultured in a 35-mm cell culture dish in DMEM with 10% FBS (GIBCO BRL USA, Grand Island, NY, USA), which was then replaced with OPTI-MEM medium (GIBCO, Invitrogen) after reaching about 75% confluence. Then, 7.5 μl Lipofectamine 3,000 was added to 250 μl OPTI-MEM (GIBCO, Invitrogen) medium and allowed to rest for 5 min, after which 2.5 μg of the plasmid was added and gently shaken for 5 sec to allow for even distribution of the plasmid in solution. After standing for 5 min, the mixture was slowly added dropwise to a centrifuge tube containing Lipofectamine 3,000, gently shaken for 5 sec, and then allowed to rest for 10 min before the mixture was transferred to cell culture medium by pipetting. The cell culture dish was gently shaken to uniformly distribute liposomes, and then the mixture was transferred to the cell incubator.

Production of lentivirus

Next, $1 \times 10^{6.0}$ 293T cells were cultured in a 10-cm cell culture dish with 6 ml DMEM plus 10% FBS. The mixture of lenti-CRISPR V2-gRNA, pCMV-VSV-G, and pSPAX2 (4:3:2) totaling 22.5 μg was gently mixed with 200 μl OPTI-MEM in a 1.5-ml centrifuge tube, and 67.5 μg polyetherimide (PEI) was mixed with 200 μl OPTI-MEM culture broth in another 1.5 ml centrifuge tube (PEI:plasmid=3:1). After standing for 5 min, the plasmid mixture was added dropwise to the PEI mixture and mixed by gentle shaking. After standing for 10 min at 25°C, the mixture was uniformly added to the culture medium. The cell culture dishes were gently shaken to evenly distribute liposomes in each dish. After transfection, the culture dish was placed in a cell incubator for 12 hr. The culture medium was then replaced and the dish was incubated for another 24 hr, after which the cell culture supernatant containing lentivirus was harvested. Then, 6 ml DMEM containing 10% FBS was added, and the cells were incubated again, for 24 hr. The supernatant was harvested at 4°C, centrifuged at 1,200 × g for 10 min, and filtered through a 0.45 μm filter. The lentivirus titers were determined and used for cell infection ($1 \times 10^{7.0}$ PFU/ml).

Construction of the ST cells stably expressing the CRISPR/Cas9 system

One ml of the lentiviral solution ($1 \times 10^{7.0}$ PFU/ml) was added to cultured ST cells when the cell confluence reached about 80%, and infection was repeated 4 times (24 hr per repetition). The cells were passaged for 4 generations and screened using puromycin (3 μg/ml) to obtain stable cell lines. The genomic DNA was isolated using a Takara MiniBEST Universal Genomic DNA Extraction Kit Ver. 5.0 (Takara China). The sgRNA in the host cell genome was determined using polymerase chain reaction (PCR) and sequencing, and Cas9 protein expression was verified using western blotting.

Table 1. gRNA for pseudorabies virus (PRV)

sgRNA name	Sequence	Target locus
PRV-gE-F	5′-caccgGCCGGCGACGATGACCTCGA-3′	122841–122861
PRV-gE-R	5′-aaacTCGAGGTCATCGTCGCCGGCc-3′	
PRV-TK-F	5′-caccgTGCCCGAGCCGATGGCGTAC-3′	59515–59535
PRV-TK-R	5′-aaacGTACGCCATCGGCTCGGGCAc-3′	
PRV-gC-F	5′-caccgTCGACCACGACGGCGCTCGA-3′	96423–98443
PRV-gC-R	5′-aaacTCGAGCGCCGTCGTGGTCGAc-3′	

Table 2. The primers for polymerase chain reaction (PCR), quantitative-PCR (q-PCR), and sequencing

Primer name	Sequence	Product length
PRV-gE-F	5′-AAAAGGTGGTGTTTGCATAATT-3′	2,697 bp
PRV-gE-R	5′-TCGGTGGTGATGTAGAACG-3′	
PRV-TK-F	5′-TCGTAGAAGCGGTTGTGG-3′	1,324 bp
PRV-TK-R	5′-CGACCAGGACGAACAGG-3′	
PRV-gC-F	5′-ATGGCCTCGC TCGCGCGTGC-3′	1,464 bp
PRV-gC-R	5′-CGCC GGTCCGCGCT GTGA-3′	
PRV-gE-F	5′-TGCCGCGGCTCCGGCGCGAG-3′	200 bp (q-PCR)
PRV-gE-R	5′-CGCACCTTCGCCCCGAGCAC-3′	
PRV-TK-F	5′-CGCGATCACCGCCGCGGCCCG-3′	130 bp (q-PCR)
PRV-TK-R	5′-TCATGCAGCGGCCCGGCG-3′	
PRV-gC-F	5′-CGAGACCGAGGGCGTCTACAC-3′	171 bp (q-PCR)
PRV-gC-R	5′-GCCCATCATCAGCGCCTGC-3′	
V2-sgRNA-F	5′-GTGAATAGAGTTAGGCAGGGAT-3′	797 bp
V2-sgRNA-R	5′-CCAACTTCTCGGGGACTG-3′	
ST-GAPDH-F	5′-GGTCGGAGTGAACGGATTT-3′	245 bp
ST-GAPDH-R	5′-ATTTGATGTTGGCGGGAT-3′	
PRV-IE180-F	5′-AGGTCTTCTGGGTGCGAT-3′	102 bp
PRV-IE180-R	5′-ACATCGGGGACGCCAT-3′	

PRV infection

The ST cells stably expressing CRISPR/Cas9 were infected with PRV ($1 \times 10^{4.0}$ TCID$_{50}$). The infected cells were collected when 80% cells showed obvious cytopathic effects (CPE). After three freezing and thawing cycles, genomic viral DNA was extracted from the infected cells, and then amplified by PCR using a series of primers and PRV target sequences (gC, gE, and TK). All editing sites were verified via sequencing.

DNA and primer sequences, PCR, sequencing, and quantitative RT-PCR

All gene sequences and PRV obtained from NCBI were analyzed using BioEdit software, primers and DNA sequences were synthesized by Shanghai Sangon Biotech, China, and DNA sequencing was performed by Fujian BioSune Biotech, China (Tables 1 and 2). The various sgRNAs were designed using the online CRISPR Design Tool (http://crispr.mit.edu).

PCR was performed in 20 μl composed of 7.2 μl ddH$_2$O, 10 μl PrimeSTAR$^®$ Max DNA Polymerase (Takara China), 0.4 μl forward primer, 0.4 μl reverse primer, and 50–100 ng (2 μl) of the viral or cellular genomic template. The PCR protocol was performed as follows: pre-denaturing at 98°C for 5 min followed by 35 cycles of denaturing at 98°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min. Then, the reaction was extended at 72°C for 10 min and ended at 4°C. The resulting PCR products were gel-purified and used for further sequencing.

For RT-qPCR analysis, total RNA was extracted with TRIzol (Sigma) and reverse transcription was performed using the PrimeScript^{TM} RT Reagent Kit with gDNA Eraser (Takara China). The composition of q-PCR reagents is as follows: 10.0 μl TaqMan$^®$ fast Advanced Master Mix (2×), 1.0 μl 1×TaqMan$^®$ Assay primer/probe (20×), 2.0 μl 1× cDNA template, 7.0 μl Nuclease-free water, with total volume per reaction amounting to 20 μl. The procedure was as follows: pre-denaturation at 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. The protocol for the melting curve stage was as follows: 95°C for 15 sec, 72−90°C for 35 cycles and each cycle increased by 0.1°C. PCR was performed on the ABI StepOnePlus fast Real-Time PCR system (Applied Biosystems, Watertown, MA, USA), and the changes in expression were calculated using the 2-$^{\Delta\Delta}$CT method.

Western blot analysis

The cell samples were collected and lysed with a protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 5% glycerol, and 2% SDS. The total protein concentration was determined using Coomassie Plus (Pierce Co., Ltd., Kenilworth, FL, USA). Equal amounts of protein were electrophoresed on 10% Tricine-SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were then blocked overnight at 4°C using a blocking buffer. Next, hybridization was performed with the corresponding primary antibodies followed by IRDye 800CW secondary antibody (1:1,000), and these were then visualized using the Odyssey CLx Western Blot Detection System (LI-COR, Lincoln, NE, USA). GAPDH expression was used as the endogenous control.

Viral plaque purification

Both the genetically modified and the original PRV strains ($1 \times 10^{3.0}$ TCID$_{50}$) were inoculated into ST cell monolayers in 10 cm culture plates. After incubation for 4 hr at 37°C, the cells were washed twice with PBS and cultured in DMEM plus 2% FBS.

After obvious cytopathic effects were observed via a microscope, the monolayers were washed twice with PBS, and the individual plaques were obtained using pipette tips.

Effects of mutated viruses in cells

Plaque counting and measurement: The ST cell monolayers were infected with wild type and genetically modified viruses, including PRV, PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gE$\Delta$TK ($1 \times 10^{3.0}$ TCID$_{50}$). After incubating for 4 hr at 37°C, the cells were washed twice with PBS and cultured in DMEM with 2% FBS for 24 hr. The cell morphologies were observed under a microscope, and morphologies of each group were recorded in 6 fields of view. In each field, plaques were counted and measured. The total number, average number, and size of plaques are shown in Fig. 2.

Comparison of plaque size: In 6-well plates, $1 \times 10^{6.0}$ cells, 2 m*l* DMEM plus 10% FBS, and $1 \times 10^{3.0}$ TCID$_{50}$ viral solution were added to each well. The viruses used for ST cell infection included PRV, PRV-ΔgC, PRVΔTK, and PRVΔgC\gE$\Delta$TK ($1 \times 10^{3.0}$ TCID$_{50}$). The control group did not contain any virus. We set up 4-time gradients at 12, 24, 36, and 48 hr; viruses were collected every 12 hr and the viral titers were determined. We used a phase-contrast microscope for continuous observation of the amplification of individual plaques to compare their infectious ability and acquire images. To obtain the plaque amplification curves, $1 \times 10^{5.0}$ cells were cultured in DMEM plus 10% FBS to reach 80% confluence, and then infected with $1 \times 10^{2.0}$ TCID$_{50}$ viruses; culturing continued for 12–15 hr. The plaques were selected and imaged every 30 min.

Animal experiments and serum neutralizing test

Four-week-old BALB/c mice were divided into various groups (5 mice per group). To evaluate the 50% lethal dose (LD$_{50}$), four groups of mice were infected with serial dilutions of the viruses (PRV, PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gE$\Delta$TK). Three other groups of mice were vaccinated with different doses of PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gE$\Delta$TK (5 mice per group) by administering 100 μl injection to the left hind leg. Fourteen days after immunization, the mice were challenged with 1×10^5 TCID$_{50}$ PRV wild type strains.

Twenty-four piglets, free of PRV, PCV2, PRRSV, CSFV, and PPV, were randomly divided into 4 groups and vaccinated with DMEM, PRV-ΔgC\gE$\Delta$TK ($1 \times 10^{6.0}$ TCID$_{50}$ once), PRV-ΔgC\gE$\Delta$TK ($1 \times 10^{6.0}$ TCID$_{50}$ twice), or commercial SA215 vaccine ($1 \times 10^{6.0}$ TCID$_{50}$); the indicated second doses were given after 7 days. At 28 days post-vaccination, the piglets were challenged with $1 \times 10^{5.0}$ TCID$_{50}$ PRV and PRV variant (isolated in our lab in 2017). After the challenge, the pigs were observed for 21 days and scored twice daily for clinical signs of the disease.

The serum was collected at 28 days post first vaccination to determine seroconversion via ELISA. PRV-specific gB antibody was evaluated using a commercial ELISA kit, according to manufacturer's directions (IDEXX, Westbrook, ME, USA), and the antibody titers were tested using the serum neutralizing test (SNT). In brief, anterior vena cava blood was collected from the pig and maintained at 37°C for 1 hr. The serum was centrifuged at $5,000 \times g$ for 5 min for antibody detection, then inactivated at 56°C for 30 min, and finally diluted with sterilized saline (our laboratory preparation). The same volume of 200 TCID$_{50}$/0.2 m*l* viral solution (PRV) was then added to each dilution. After mixing and incubating at 37°C for 1 hr, the virus–serum mixture was inoculated into Vero cells (with each dilution tested in 3 wells) followed by incubation at 37°C for 2 hr. Then, the virus–serum mixture was replaced with RPMI 1640 medium containing 2% FBS, 100 U/m*l* penicillin, and 100 g/m*l* streptomycin, and the culture was incubated at 37°C under 5% CO$_2$. At the same time, control wells with serum and virus-positive were set up. The CPEs of the cells were recorded after 5 days of culture. The neutralizing antibody titer was calculated using the Reed-Muench method.

All animal experiments were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals and approved by the Fujian Provincial Office for Managing Laboratory Animals, and were overseen by the Fujian Normal University Animal Care and Use Committee (ethics license number IACUC-2016Y0027).

Histopathology and immunohistochemistry analysis

To perform histopathological analysis, forty female BALB/c mice (8 per group) were subcutaneously inoculated with $10^{3.5}$ TCID$_{50}$ PRV, PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gE$\Delta$TK, while the control group mice were inoculated with DMEM. Two weeks later, the mice were sacrificed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the Research Ethics Committee, Fujian Normal University, Fuzhou, China. Then, the mice brain tissues were fixed with 4% paraformaldehyde solution at 25°C for 24 hr, followed by embedding them in 30% sucrose according to previously described [30]. The brain tissues were cut into 9 μm sections using a microtome and stored in a refrigerator at −80°C. The sections were removed and incubated at 25°C for 15 min, and then washed 4 times with PBS. Tissue sections were then incubated for 10 min with PBS/Triton X-100 (0.3%) (Merck KGaA, Darmstadt, Germany), followed by 2 hr incubation with 2% bovine serum albumin in PBS. Sections were then incubated with mouse monoclonal antibodies against human β-tubulin (Abcam, Cambridge, MA, USA) overnight at 4°C, washed 3 times with PBS, and further incubated with fluorescent-conjugated donkey anti-rabbit IgG secondary antibody (Thermo Scientific) for 2 hr at 37°C. To visualize the nuclei, sections were stained with 4,6-diamino-2-phenylindole (DAPI; Merck & Co., Fort Pierce, NJ, USA) and treated with an autofluorescence eliminator reagent (Merck & Co.). Images were taken with a Zeiss fluorescence microscope (Zeiss & Co., Ohringen, Germany) and analyzed using the NIH Image software.

Statistical analysis

The significant differences in the animal experiments were analyzed using Tukey's test and the GraphPad Prism (version 8.0.1) software (San Diego, CA, USA). Differences were considered statistically significant when $P<0.05$.

RESULTS

Construction of stably expressed Cas9 and sgRNA

To establish stable expression of the CRISPR/Cas9 system, sgRNAs (Fig. 1a) targeting TK, gC, and gE genes were inserted into the lenti-CRISPR V2 plasmid under the U6 promoter. This plasmid, along with the PCMV-VSV-G and sPAX2 plasmids, was used to produce the recombinant lentivirus that was used to infect ST cells, followed by a puromycin screen. The stable expression of Cas9 protein was verified using PCR, sequencing, western blotting, and an anti-FLAG antibody (Fig. 1b).

Deletion of gE, TK, and gC in PRV by stable expression of Cas9 protein

Cells stably expressing Cas9 and viral sgRNAs were selected using their resistance to puromycin. Twelve hours after viral infection, the cells were collected and subjected to PCR and sequencing to determine whether the system was valid, based on overlapping peaks shown in the sequencing data. Then, 3 or 4 rounds of infection were conducted. In order to obtain pure mutant strains, we performed plaque screening, followed by PCR and sequencing verification (as described in the methods section). The mRNA level of gE, TK, and gC on the PRV-ΔgC\gEΔTK group was significantly lower than that of the wild type virus (Fig. 1c). The plaque screening and sequencing data showed that gE, TK, and gC mutants were successfully isolated, with deletions of 5, 4, and 5 bp, respectively (Fig. 1d).

Biological characterization of PRV-ΔTK, PRV-ΔgC, and PRV-ΔgC\gEΔTK

To further characterize the mutant viruses with deletions in the gE, TK, and gC genes, we selected 3 mutant viruses (PRV-ΔTK, PRV-ΔgC, PRV-ΔgC\gEΔTK) isolated from the plaque screening for further validation. The plaque morphologies of the above-mentioned mutant strains differed from those of the wild type viral strains when grown in ST cell monolayers, and the number and size of the mutant viral plaques were both significantly reduced as compared to that of the wild type PRV (Fig. 2-1a, 2-1b). Reduction in the plaque size of mutant strains became more apparent following longer growth times. These data strongly suggest that TK, gC, and gE genes play a critical role in PRV viral replication via an unknown mechanism.

PRV-ΔTK, PRV-ΔgC, and PRVΔgC\gEΔTK growth on ST cells

PRV-ΔTK, PRV-ΔgC, PRVΔgC\gEΔTK, and PRV were simultaneously inoculated into ST cell monolayers. The data showed that PRV-ΔTK, PRV-ΔgC, and PRV-ΔgC\gEΔTK grew at a slower rate than PRV Fa, while PRV-ΔgC\gEΔTK grew the slowest (Fig. 2-2a). However, over time, the growth gap between the strains gradually narrowed. At 12 and 24 hr post infection, PRV-ΔTK, PRV-ΔgC, and PRV-ΔgC\gEΔTK titers were significantly lower than those of PRV Fa, and there was no significant difference between them (PRV-ΔTK, PRV-ΔgC, and PRV-ΔgC\gEΔTK). At 36 hr, the titer of PRV-ΔgC was significantly ($P<0.05$) higher than that of PRV-ΔTK and PRV-ΔgC\gEΔTK. After 48 hr of infection, PRV-ΔTK, PRV-ΔgC, and PRV-ΔgC\gEΔTK titers were consistent with those of PRV Fa (Fig. 2-2b). These data suggest that the strains with deleted genes possess a relatively sound
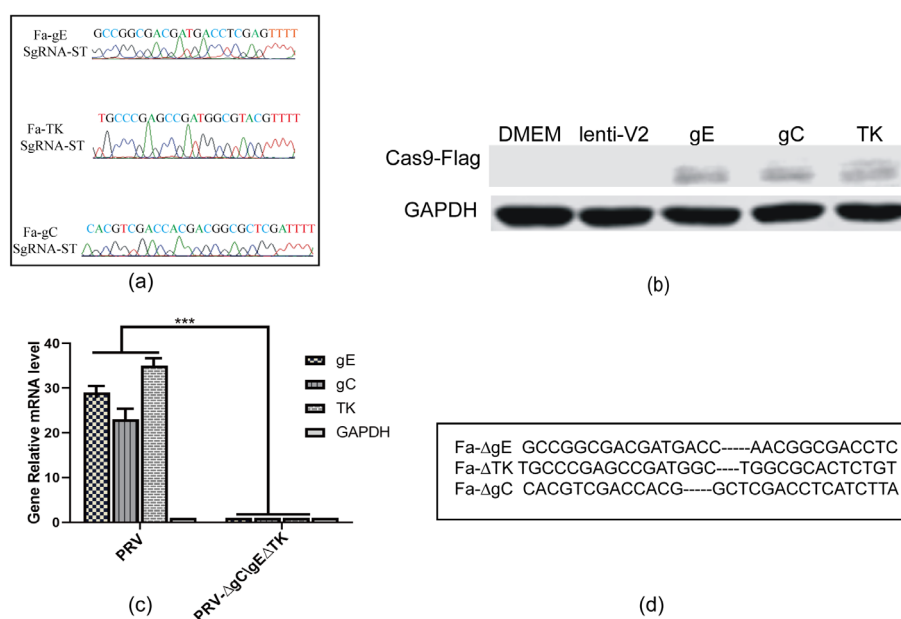

Fig. 1. Identification of swine testis (ST) cell-Cas9^{+}/sgRNA[+] and virus knockout strains. (a) Design of sgRNAs for targeting virulence-associated genes (TK, gC, gE) of pseudorabies virus (PRV) Fa. (b) Verification of Cas9 expression in the ST-Cas9/sgRNA system expressed in the host ST cells using western blotting. (c) The mRNA level of gC, gE, and TK of PRV-ΔgC\gEΔTK and PRV Fa. (d) Sequencing analyses confirming the presence of mutant gC, gE, and TK genes of PRV in host cells (*** $P<0.001$, Tukey's test).
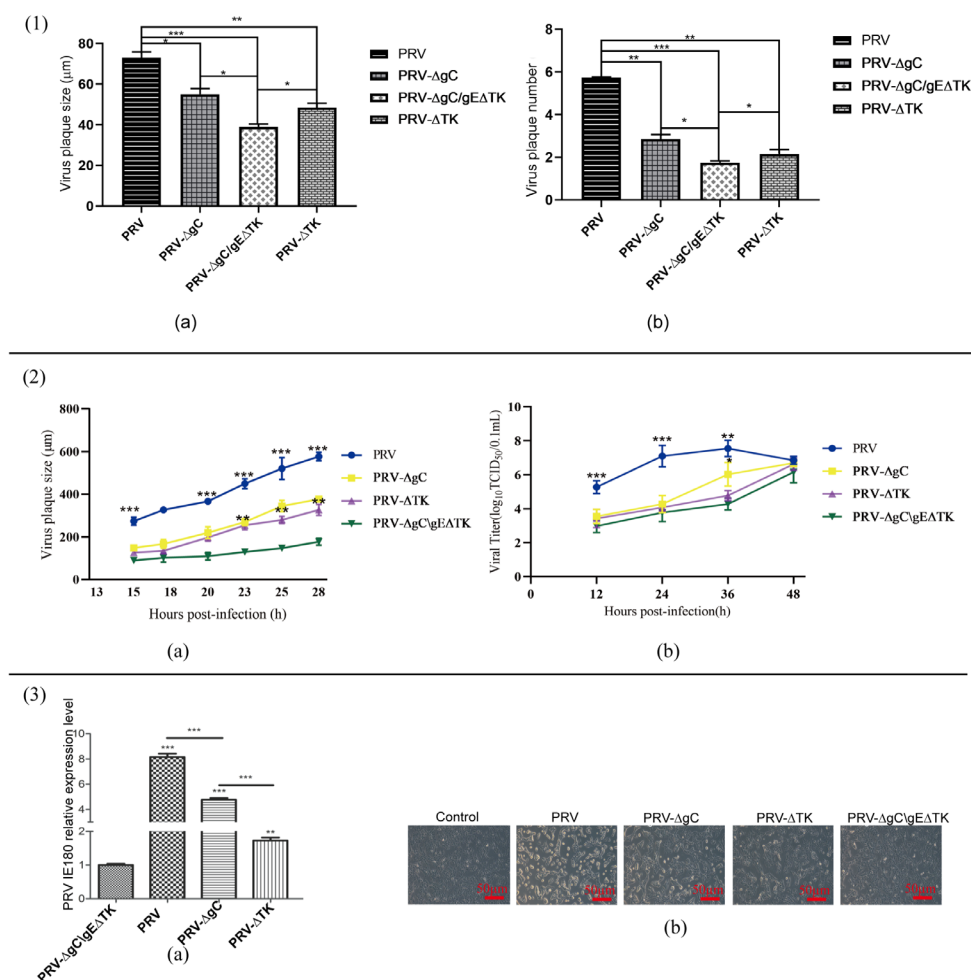
Fig. 2. Verification of the virulence of pseudorabies virus (PRV), PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gEΔTK at the cellular level. 2-1a) Morphology of plaques in the swine testis (ST) cell monolayer. 2-1b) The number of plaques in ST cell monolayers infected with PRV, PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gEΔTK. 2a) Plaque morphologies present in ST cell monolayers infected with PRV, PRV-ΔgC, PRV-ΔTK, and PRV-gC\gEΔTK viruses. 2b) The titers of PRV, PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gEΔTK viruses in different infection times. 3a, 3b) PRV IE180 relative expression level and plaque morphologies after infection with PRV, PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gEΔTK viruses. Statistical analyses were performed using Tukey's test and the data are presented as mean ± SD (n=6, *$P<0.05$, **$P<0.01$, ***$P<0.001$). Data was processed using GraphPAD prism 8.0.1 and CorelDRAW12 software.

proliferative ability for producing sufficient immunogenicity for further vaccine development.

The immediate early 180 (IE180) gene is the only IE gene of PRV, possessing the important role of viral replication processing. As long as the IE180 gene is transcribed and translated into the IE180 protein, transcription and replication of its downstream genes should be activated. Consequently, Fig. 2-3a and 2-3b show that the deletion of gC, gE, and TK significantly decreased the transcription of IE180 mRNA, while it was not entirely blocked. It was observed that PRV with gC, gE, and TK deletions grew slowly.

The safety and immunogenicity of PRV-ΔTK, PRV-ΔgC, and PRV-ΔgC\gEΔTK in mice

Vaccine safety is a primary factor taken into consideration during vaccine development. Therefore, PRV-ΔgC\gEΔTK was assessed for its safety as a vaccine candidate using mice. Given that the mutant PRV genes caused a decrease in the viral propagation capacity (see above), we characterized the virulence of these attenuated viral strains in infected mice. Previous studies have shown that mice could be used as an animal model to effectively address pathologic injury caused by acute PRV infection. The median lethal dose, LD_{50}, represents viral virulence in virology, where a lower LD_{50} is indicative of increased toxicity. Here, mice were injected with different doses of PRV Fa, and the LD_{50} dose for PRV was $1 \times 10^{3.8}$ $TCID_{50}$ (Fig. 3a). In contrast, when inoculated in mice, the attenuated mutant viruses had higher LD_{50} doses. The LD_{50} of PRV-ΔgC was $1 \times 10^{4.3}$ $TCID_{50}$, while those of PRV-ΔgC\gEΔTK and PRV-ΔTK were both greater than $1 \times 10^{6.0}$ $TCID_{50}$ (Fig. 3b, 3c, 3d), showing a clear decrease in viral infectivity. These data suggest that the virulent effects of attenuated PRV were indeed weakened by the deletion of gC or TK and gE in PRV, through genetic editing.

To further test the protective effect of gene-deletion in viruses, mice were vaccinated with different doses of PRV-ΔgC,
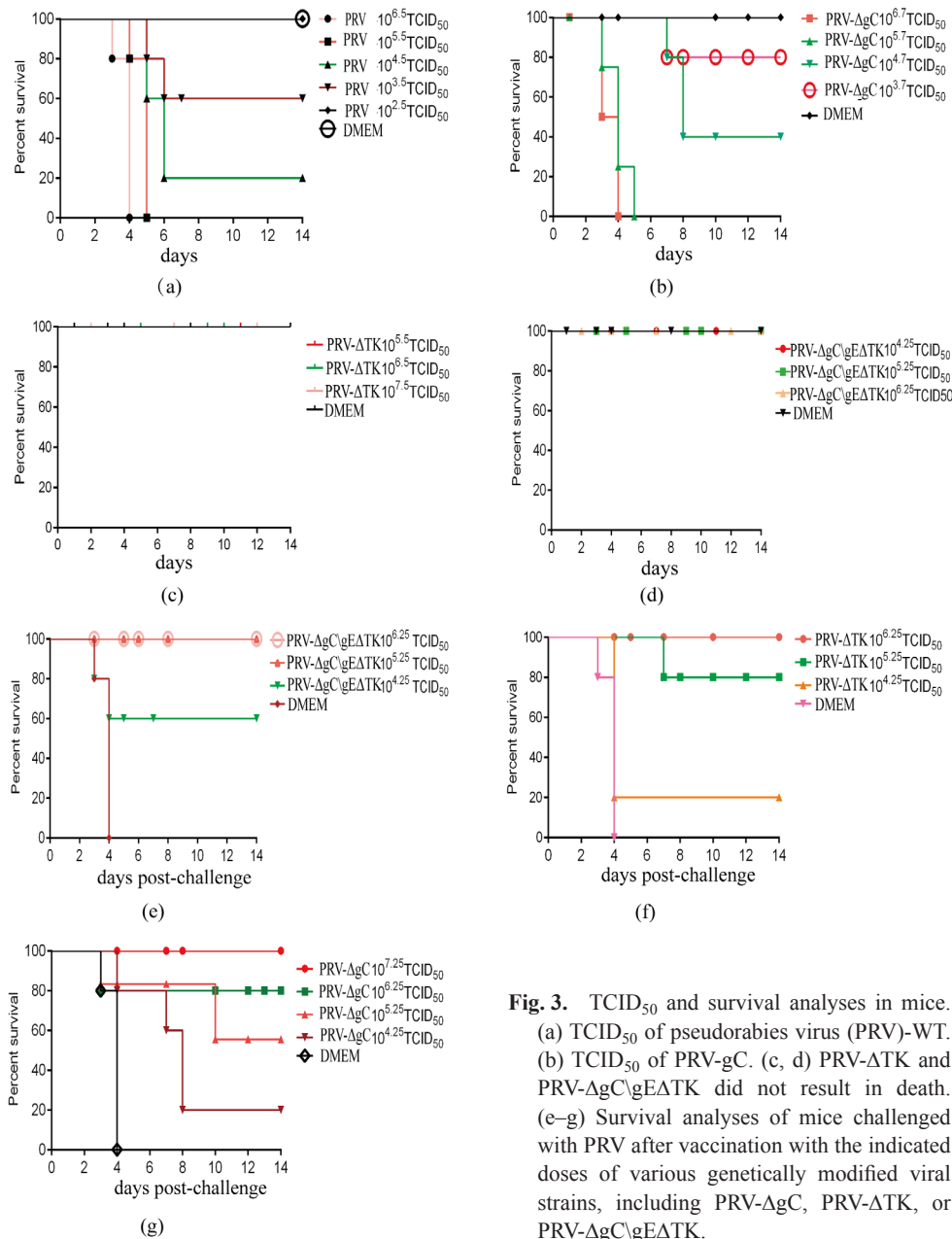
Fig. 3. TCID$_{50}$ and survival analyses in mice.
(a) TCID$_{50}$ of pseudorabies virus (PRV)-WT.
(b) TCID$_{50}$ of PRV-gC. (c, d) PRV-ΔTK and
PRV-ΔgC\gEΔTK did not result in death.
(e–g) Survival analyses of mice challenged
with PRV after vaccination with the indicated
doses of various genetically modified viral
strains, including PRV-ΔgC, PRV-ΔTK, or
PRV-ΔgC\gEΔTK.

PRV-ΔTK, and PRV-ΔgC\gEΔTK. At 28 days post-immunization, these mice were challenged with $1 × 10^{5.0}$ TCID$_{50}$ PRV. The data showed that vaccination with PRV-ΔgC\gEΔTK ($1 × 10^{6.25}$ TCID$_{50}$) and PRV-ΔTK ($1 × 10^{6.25}$ TCID$_{50}$) provided complete protection against infection by the PRV strain, and no severe PR symptoms were observed (Fig. 3e, 3f, and 3g). In contrast, mice in the control group displayed apparent symptoms, including anorexia and intense pruritus, which led to lesions due to scratching and self-mutilation. Nervous signs became more pronounced and lack of coordination ensued, eventually resulting in paralysis.

To verify the virulent effects, forty female BALB/c mice (8 per group) were subcutaneously inoculated with $10^{3.5}$ TCID$_{50}$ PRV, PRV-ΔgC, PRV-ΔTK, and PRV-ΔgC\gEΔTK, while the control group mice were inoculated with DMEM. Two weeks later, the dead and surviving mice were subjected to histological analyses. In the PRV group, Purkinje cells between the molecular and granular layers were distributed over irregular, widely spaced intervals (Fig. 4a). Nerve microtubules in the superficial layer of the cerebellum suffered severe damage, as indicated by broken cytoarchitectures (Fig. 4b). Immunohistochemical analyses revealed severe pathological damage in the brain, such as obvious meningeal inflammation and neuronal loss with clear signs of apoptosis in the granular layer of the cerebellum, as indicated by TUNEL assays (Fig. 4c). However, in the PRV-ΔgC and PRV-ΔTK groups, the nerve microtubules in the superficial layer of the cerebellum suffered from little damage, as indicated by broken cytoarchitectures, while the PRV-ΔgC\gEΔTK and control groups showed no obvious damage to the cerebellar layer or neurons (Fig. 4a–c).
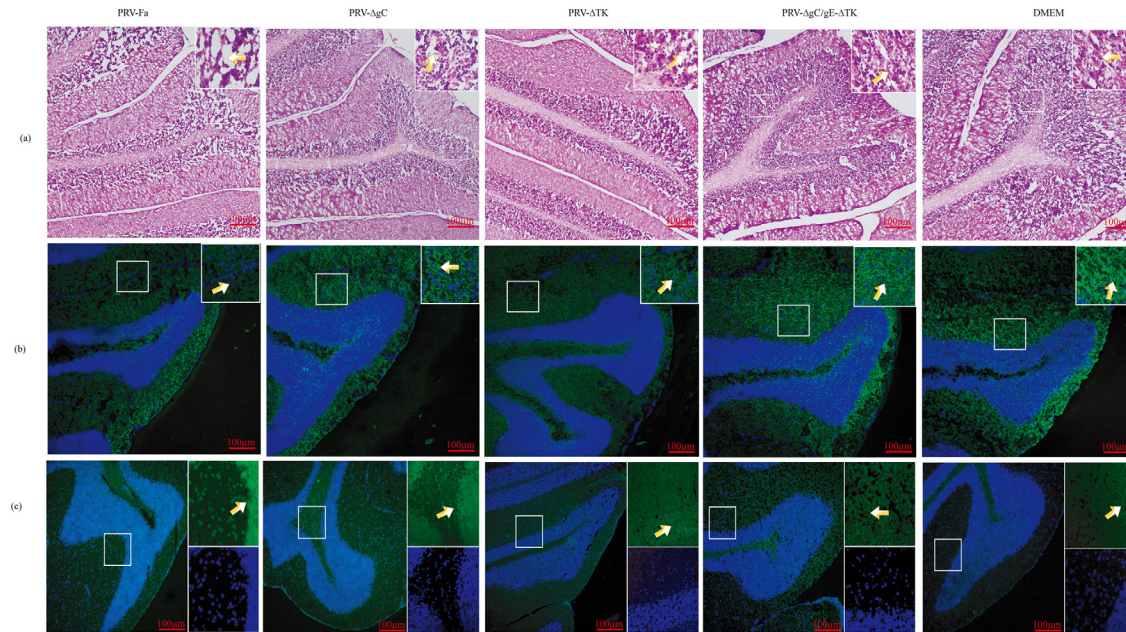
Fig. 4. Pathological changes in the brain caused by pseudorabies virus (PRV), PRVΔgC, PRVΔTK, and PRV-ΔgC\gEΔTK in mice. (a) Pathological changes in the cerebella of mice caused by infection with PRV, PRVΔgC, PRV-ΔTK, or PRVΔgC\gEΔTK were observed using hematoxylin and eosin staining. (b) Tubulin distribution and morphology in mouse cerebella were observed using immunofluorescence. (c) Neuronal apoptosis in the cerebellar sections detected with TUNEL (TdT-mediated dUTP Nick-End Labeling). The enlarged area is indicated by yellow arrows, and the red bar represents 200 μm. Data was processed using CorelDRAW12 software.

PRV-ΔgC\gEΔTK strains provide full protection in piglets

PRV is a member of the Alphaherpesvirinae subfamily, and its infection can lead to one of the most devastating infectious diseases in swine. To test the vaccinal and protective efficacy of the PRV-ΔgC\gEΔTK vaccine in pigs, serum samples were collected from animals 28 days post-challenge and tested for gB-specific antibodies using PRV antibody detection kits. The data showed that anti-gB antibody increased significantly in vaccinated (once and twice) piglets (Fig. 5a), and the neutralization titers of the vaccinated group were significantly higher than those of the control group against PRV ($P<0.001$), and PRV-ΔgC\gEΔTK vaccinated group exhibited neutralization titers similar to SA215 against PRV (Fig. 5b).

At 28 days post-immunization, the pigs were challenged with $1 \times 10^{5.0}$ TCID$_{50}$ PRV wild type and PRV variant strains. Three vaccinated groups showed similar symptoms of depression and reduced food intake. Additionally, no neurological signs were recognized in the three groups, and all piglets remained alive. Accordingly, pigs vaccinated with DMEM developed typical PR symptoms, such as high fever, depression, anorexia, respiratory distress, vomiting, and trembling, and three piglets died; each on the 2, 5, and 8 days post-vaccination. These results demonstrated that PRV-ΔgC\gEΔTK is a safe and effective candidate vaccine for pig herds.

DISCUSSION

Due to the prevalence of a novel variant of PRV in recent years, commercially available PRV vaccines, particularly the Bartha-K61 vaccine, did not provide highly effective protection, causing major losses to local farmers [2, 14, 30]. Therefore, the development of a live-attenuated vaccine for the PRV variant is currently crucial for controlling PRV. In this study, we constructed and evaluated PRV-ΔgC\gEΔTK as a live vaccine candidate.

A live vaccine candidate requires a balance of safety, immunogenicity, and broad protection. The PRV strain of Fa was the first to be reported in 2000 [4], leading to the prevalence of PR in China as well as major economic losses to the pig industry. However, phylogenetic analyses have suggested that most of the newly isolated PRV strains in China in recent years originated from evolution of the parental PRV strains, some of which are very closely related, such as HuB-China-2016, GD-QY-2010, and GD-JM-2015 [1, 6, 21, 27, 28,]. Therefore, PRV Fa is a typical strain suitable as a template for vaccine development. Some previous reports have indicated that a gene-deficient strain derived from the PRV Fa strain, SA215 (gE⁻/gI⁻/TK⁻), which has high immunogenicity and long-term immunity, and was developed as a commercial live-attenuated vaccine, could afford adequate protection against various PRV strains [10, 26].

Genes crucial for virulence but inessential for replication are potential targets for PRV live vaccine development. It is known that gI/gE forms a complex and is responsible for neurotropism and reactivation. Therefore, most PRV vaccine candidates exhibit gI/gE deletion, which is most desirable for attenuation and differential diagnostic tests. In PRV Fa, the deletion of gE breaks the
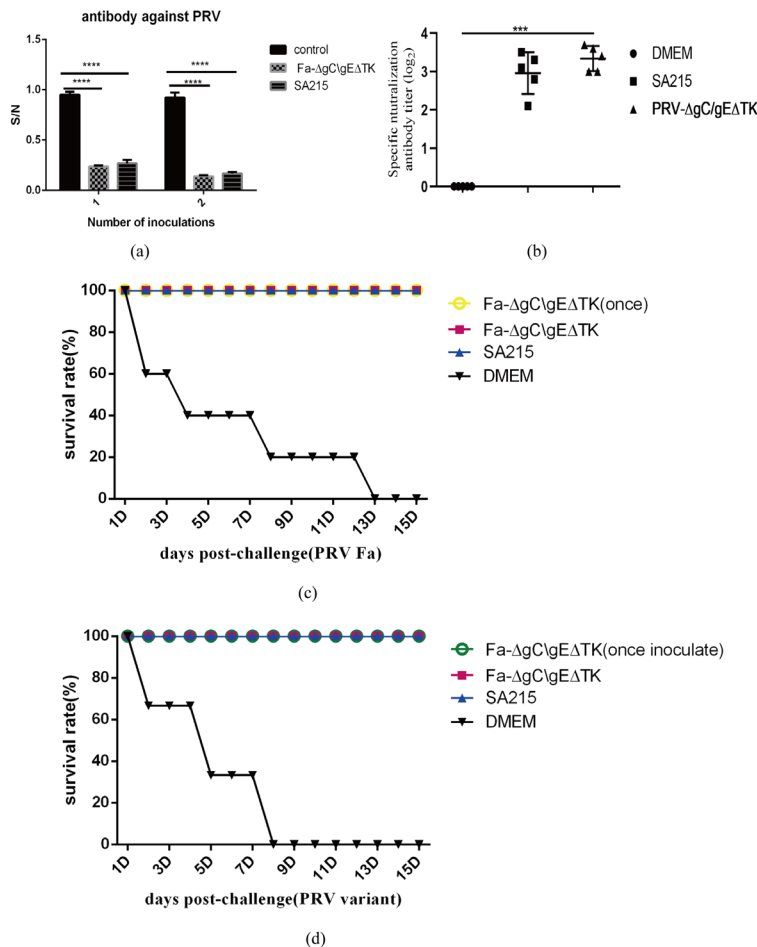
Fig. 5. Protective efficacy of attenuated live vaccines against pseudorabies virus (PRV) challenge in pigs. (a) Anti-PRV seroconversion in pigs before and after challenge. Sera were collected at 0 and 28 days post-vaccination. Commercial ELISA kits were used to test anti-PRV gB antibody. The S/N ratio was calculated as the sample OD450 divided by the negative control OD450. S/N <0.6, positive; S/N between 0.6 and 0.7, inconclusive; S/N >0.7, negative. (b) Neutralizing antibodies against PRV were tested in 96-well plates (a, b: Data was analyzed using Tukey's test). (c) Survival rate with PRV challenge. (d) Survival rate with PRV variant challenge.

function of the gI/gE complex and may be safe enough for newborn piglets; therefore, gI deletion is worth considering [15, 17, 32]. In addition, gC and TK were also involved in the invasion of the nervous system, as gC can affect virus release, and TK is associated with viral replication in non-dividing cells [29]. Therefore, gC deletion may be more necessary for the development of an attenuated vaccine. Our studies also confirmed that the plaque size and numbers reduced significantly with gC deletion compared to those with PRV Fa. In addition, the deletion of gC genes significantly alleviated viral damage to the brain, and LD_{50} was significantly upregulated, while IE180 mRNA was also significantly downregulated. The results indicated that deletion of gC will slow PRV replication and improve the safety of candidate vaccine development.

In this study, we also tested the safety of the attenuated virus in 4-week-old mice. Mice were injected with different doses of wild type PRV. The PRV LD_{50} dose was $1 \times 10^{3.8}$ $TCID_{50}$. In contrast, the LD_{50} of PRV-ΔgC was $1 \times 10^{4.35}$ $TCID_{50}$, while those of PRV-ΔgC\gEΔTK and PRV-ΔTK were greater than $1 \times 10^{6.0}$ $TCID_{50}$, showing a clear decrease in viral infectivity. These results suggest that PRV-ΔgC\gEΔTK is safe for mice. Furthermore, the virulence-weakening assay also indicated that the deletion of TK and gC in PRV clearly reduced cerebral lesions and particularly reduced the destruction of tubulins in the central nervous system. Thus, TK and gC may also be essential for the persistence of PRV in the neural tissues of mice [29]. We also showed that mortality rate was greatly reduced when mice were infected with a gC mutant strain (PRV-ΔgC) than a wild type virus. Therefore, we believe that PRV-ΔgC\gEΔTK could be suitable for use as a candidate for vaccine production.

The efficacy evaluation demonstrated that a single inoculation of $10^{5.25}$ $TCID_{50}$ PRV-ΔgC\gEΔTK was capable of providing full protection to mice and pigs against challenge with $10^{5.0}$ $TCID_{50}$ PRV Fa. Conversely, in the DMEM-vaccinated group, no mice survived after a challenge. This further demonstrated that immunization with the gC, gE, and TK triple-gene-deletion strain with a lower $TCID_{50}$ afforded sufficient protection for the mice.

In growing pigs, the gB antibody generated by the commercial Bartha-K61 vaccine demonstrated a substantially lower protection against PRV, but the live PRV-ΔgC\gEΔTK vaccine stimulated high levels of gB antibody against PRV, which also elicited similar level of neutralizing antibody to SA215 strain [11]. These results are consistent with previous reports that suggested that recent genomic comparisons showed no significant genetic variation between PRV strains and PRV variants, and all isolated PRV variants are highly homologous and clustered within the same genotype as the PRV Fa strain. In summary, the newly developed PRV-ΔgC\gEΔTK strain was validated as a vaccine candidate of PRV, and offered protection against most variants in China.

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