Generation and Efficacy Evaluation of a Recombinant Pseudorabies Virus Variant Expressing the E2 Protein of Classical Swine Fever Virus in Pigs

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Classical swine fever (CSF) is an economically important infectious disease of pigs caused by classical swine fever virus (CSFV). Pseudorabies (PR), which is caused by pseudorabies virus (PRV), is another important infectious disease of pigs and other animals. Coinfections of pigs with PRV and CSFV occur occasionally in the field. The modified live vaccine Bartha-K61 strain has played an important role in the control of PR in many countries, including China. Since late 2011, however, increasing PR outbreaks caused by an emerging PRV variant have been reported in Bartha-K61-vaccinated swine populations on many farms in China. Previously, we generated a gE/gI-deleted PRV (rPRVTJ-delgE) based on this PRV variant, which was shown to be safe and can provide rapid and complete protection against lethal challenge with the PRV variant in pigs. Here, we generated a new recombinant PRV variant expressing the E2 gene of CSFV (rPRVTJ-delgE/gI-E2) and evaluated its immunogenicity and efficacy in pigs. The results showed that rPRVTJ-delgE/gI-E2 was safe for pigs, induced detectable anti-PRV and anti-CSFV neutralizing antibodies, and provided complete protection against the lethal challenge with either the PRV TJ strain or the CSFV Shimen strain. The data indicate that rPRVTJ-delgE/gI-E2 is a promising candidate bivalent vaccine against PRV and CSFV coinfections.

Materials and Methods

**Viruses and cells.** The PRV TJ strain (PRVTJ), a virulent PRV variant (15), and the highly virulent CSFV Shimen strain were used for PRV- and CSFV-specific neutralizing test and virus challenge. The gE- and gI-deleted mutant based on the emergent PRV variant, was safe for pigs and provided complete protection against lethal challenge with the PRV variant (17). In this study, we generated a PRV variant-based recombinant expressing the CSFV E2 protein and evaluated its safety, immunogenicity, and efficacy in pigs.

**MATERIALS AND METHODS**

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lected PRV mutants rPRVTJ-delgE and rPRVTJ-delgE/gI-EGFP were described previously (Fig. 1) (17). The CSF C-strain vaccine (lot no. 2014001) was produced by Weike Biotech Co., Harbin, China. All PRV strains were propagated and titrated in PK-15 or Vero cells, which were grown at 37°C and 5% CO2 and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA), 100 μg/ml streptomycin, and 100 IU/ml penicillin. The construction of the recombinant transfer plasmid. A universal transfer plasmid, pOK-LR (17), was used as a backbone to construct the recombinant transfer plasmid. The human cytomegalovirus (hCMV) promoter and the CSFV E2 gene were amplified with the primer pairs P1S/P1R and P2S/P2R (Table 1) from pEGFP-N1 (Clontech, USA) and pShuttle-E2 (18), respectively. To generate the transfer plasmid pOK-LR-CMV-E2, the E2 expression cassette was inserted under the control of the CMV promoter (CMV-E2) was amplified by PCR using the purified CMV and E2 fragments as the templates with primers P1S and P2R and cloned into the MluI site of pOK-LR. The recombinant transfer plasmid pOK-LR-CMV-E2 was verified by restriction enzyme analysis, sequencing, and immunofluorescence assay (IFA).

Rescue of the recombinant virus and plaque assay. The recombinant virus was rescued as previously described (17). In brief, the genomic DNA of the PRV TJ mutant rPRVTJ-delgE/gI-EGFP was extracted, digested with PacI and PmeI, and cotransfected with pOK-LR-CMV-E2 into Vero cells. The rescued virus was subjected to at least six rounds of plaque assay. During each round of plaque purification, the presence of the E2 gene and the absence of the gE gene were verified by PCR using E2-specific (P2S/P2R) and gE-specific (P3S/P3R and P4S/P4R) primer pairs (Table 1).

In order to obtain a plaque isolate with high-level expression of the heterologous protein, better propagation traits, and genetic stability, another round of purification against the recombinant virus was performed by plaque assay and 10 plaque isolates were randomly picked out. Following freezing at −80°C and thawing at 37°C three times, all 10 plaque isolates were passaged 20 times on PK-15 cells and then were identified and compared by PCR, IFA, and flow cytometry as described below.

PCR. After the 10 plaque isolates were passaged 20 times on PK-15 cells, the genomic DNA of different plaque isolates was extracted. The inserted CSFV E2 fragment was amplified by PCR with the primers P2S/P2R and sequenced to verify the stability of different plaque isolates during the passages.

Immunofluorescence assay. To check the expression of the CSFV E2 protein, PK-15 cells were infected with different plaque isolates at a multiplicity of infection (MOI) of 1 for 24 h. Cells were fixed with cold absolute ethyl alcohol for 20 min. The fixed cells were incubated with the anti-E2 monoclonal antibody (MAb) HQ06 (19) for 2 h at 37°C in a humidified chamber, followed by three washes with phosphate-buffered saline (PBS), and then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma-Aldrich, USA) for 1 h at 37°C. Following three washes with PBS, the cells were examined under a fluorescence microscope (Nikon TE200; Japan).

Flow cytometry. Ten plaque isolates were picked out and passaged 20 times on PK-15 cells. PK-15 cells were infected with different plaque isolates at an MOI of 1 for 12 h and then digested with trypsin and filtered with a 300-mesh sieve to disperse the cells. The cells were washed three times with prechilled PBS, and the viable cells were detected by trypan blue staining; then, the cells were incubated with 500 μl of the anti-E2 MAb HQ06 (diluted 1:1,000) at 37°C for 2 h. After washing three times with prechilled PBS, the cells were incubated with 500 μl of FITC-labeled goat anti-mouse IgG (diluted 1:100) at 37°C for 1 h. Following washing three times with prechilled PBS, the cells were resuspended in 500 μl of PBS. Propidium iodide (PI) staining was used to exclude nonviable cells, and 104 viable cells were included to analyze the mean fluorescence intensity.

![FIG 1](https://example.com/fig1.png)

**TABLE 1** Sequences of PCR primers used in the study

| Fragment | Primer | Sequence | Product size (bp) |
|----------|--------|----------|------------------|
| CMV      | P1S    | 5′-CGACGCGCTTAGTTATTTATAGTAATCAATT-3′ (introduced Mmul site underlined) | 704 |
|          | P1R    | 5′-CGACGCGTTTAGTTATTTATAGTAATCAATT-3′ (introduced Mmul site underlined) | 704 |
| E2       | P2S    | 5′-TCCACCGCTGCGCACCATGTTATTTAAGGAGCAGCTCGTGAAGGTTGAT-3′ | 1,210 |
|          | P2R    | 5′-GCACGCGTTTAGCTTTATACCGGGCGAGCGGATGCCCGCGCGCGTA-3′ (introduced Mmul site underlined) | |
| Partial LRa | P3S   | 5′-GATGATGTGGCGCGCGCGCGGCGGATGCCCGCGCGGAAGGT-3′ | 532 |
|          | P3R    | 5′-CTCCACCGCTGCGCACCATGTTATTTAAGGAGCAGCTCGTGAAGGTTGAT-3′ | 1,210 |
| gE       | P4S    | 5′-GGGGTTGGCAGCAGGAGACACCA-3′ | 1,897 |
|          | P4R    | 5′-ACCAGCTGACCGCGCTCAA-3′ | |

a LR, left and right homology arms.
The plaque isolate with the highest MFI was screened and named rPRVTJ-delgE/gI-E2 (Fig. 1).

Western blotting. The expression of the CSFV E2 protein in rPRVTJ-delgE/gI-E2-infected PK-15 cells was further determined by Western blotting. Infected or uninfected PK-15 cells (10^6 cells/well) were treated with

| Group | Vaccine                      | Dose (TCID₅₀ unless otherwise specified) | No. of piglets | Boost interval (wk) | Challenge with strain: |
|-------|------------------------------|------------------------------------------|----------------|---------------------|------------------------|
|       |                              |                                          |                |                     | PRVTJ                  |
| 1     | rPRVTJ-delgE/gI-E2          | 10^6                                     | 5              | 1                   | 10^6                   |
| 2     | rPRVTJ-delgE/gI-E2          | 10^6                                     | 5              | 3                   | 10^6                   |
| 3     | rPRVTJ-delgE/gI-E2          | 10^6                                     | 5              | 3                   | 10^6                   |
| 4     | rPRVTJ-delgE               | 10^6                                     | 5              | 3                   | 10^6                   |
| 5     | DMEM                        | 1 ml                                     | 5              |                     | 10^6                   |
| 6     | C-strain                    | One dose                                 | 5              | 3                   | 10^6                   |
| 7     | PBS                         | 1 ml                                     | 5              |                     | 10^6                   |

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FIG 2 Screening of the recombinant virus. (A) CSFV E2 protein expression in PK-15 cells infected with different plaque isolates of the recombinant virus by IFA. PK-15 cells were either mock infected or infected with CSFV or different plaque isolates of the recombinant virus expressing the E2 protein of CSFV at an MOI of 1. The cells were fixed 48 h postinfection and were analyzed by IFA using the anti-E2 MAb HQ06 as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody. Bars, 400 μm. (B) Comparison of E2 protein expression levels in PK-15 cells infected with different plaque isolates of the recombinant virus by flow cytometry analysis. PK-15 cells were either left uninfected or infected with different plaque isolates of the recombinant virus expressing the E2 protein of CSFV at an MOI of 1 for 12 h and then digested with trypsin and filtered with mesh sieve to disperse the cells. The cells were washed with prechilled PBS three times and then incubated with MAb HQ06 and FITC-conjugated goat anti-mouse IgG, respectively. The fluorescence intensities of the CSFV E2 protein were detected by flow cytometry to evaluate the E2 expression differences of different plaque isolates. Error bars represent the standard errors of the means from two replicates. *, significant difference between plaque isolates 1 and 2 to 9 (P < 0.05); **, very significant difference between plaque isolates 1 and 10 (P < 0.001).
the lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 0.5% NP-40, 20 
µg/ml DNase I). The cell lysate was cleared of cell debris by centrifugation 
at 12,000 rpm for 5 min. Proteins in the lysate were separated by 10% 
sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) 
and subsequently transferred onto a nitrocellulose membrane (Bio-Rad, 
USA). The membrane was incubated for 1 h at room temperature with the 
anti-E2 MAb HQ06 (1:2,000) in PBS, followed by incubation with IRDye 
800CW-labeled goat anti-mouse secondary antibody (Li-Cor). The lysate 
of PK-15 cells infected with CSFV or rPRVTJ-delgE served as a positive or 
negative control, respectively. The E2 protein was visualized using the 
Odyssey infrared imaging system.

**One-step growth curve.** The growth kinetics was determined for 
rPRVTJ-delgE/gI-E2, rPRVTJ-delgE, and PRVTJ as described previously 
(17). Briefly, PK-15 cells grown in 24-well culture plates were inoculated 
with each of the above viruses at an MOI of 10 and incubated on ice for 1 h. 
Thereafter, the inoculum was replaced with prewarmed fresh DMEM and the 
infected cells were further incubated for 1 h at 37°C. Extracellular 
the virus was inactivated by low-pH treatment (20), and the cell culture was harvested 
at the indicated time points. After two freeze-thaw cycles, the cellular debris 
was removed by centrifugation and the supernatant was titrated on PK-15 
cells as described previously (15). Average titers and standard deviations of 
two independent experiments were calculated.

**Immunization and challenge of pigs.** The animal experiments were 
conducted according to the Guide for the Care and Use of Laboratory 
Animals of Harbin Veterinary Research Institute, Chinese Academy of 
Agricultural Sciences, China. Thirty-five 6-week-old healthy piglets were 
obtained from a local farm without PR and CSF history. The piglets were tested and proven to be free of CSFV and PRV by serum neutralization 
assay, enzyme-linked immunosorbent assay (ELISA), and PCR. All the 
pigs were randomly divided into seven experimental groups of 5 pigs each 
(groups 1 to 5) and 10 pigs each (groups 6 and 7), in a separate pen, were inoculated i.m. with one-dose 
C-strain vaccine or 1 ml of PBS, respectively. One week postimmunization, 
all the pigs were challenged i.m. with 10⁶ TCID₅₀ of the PRV TJ strain in the neck. Following challenge, clinical signs and rectal tempera-
tures were monitored daily throughout the experiment. At 14 days postchall-
enge (dpc), all surviving pigs in the rPRVTJ-delgE (group 4) and 
PRVTJ (group 5) groups were euthanized, and different tissues (brain, 
lung, liver, kidney, heart, spleen, bladder, tonsils, and lymph nodes) were 
collected and subjected to pathological and immunohistochemistry 
(IHC) examinations and PCR.

Three weeks after the first immunization, 10⁶- and 10⁴-TCID₅₀-
rPRVTJ-delgE/gI-E2 groups (groups 2 and 3), and the C-strain group 
(group 6), were boosted with the same vaccine and dose as those in the 
first immunization. Two weeks after the booster immunization, groups 1 to 
3 and groups 6 and 7 were each challenged i.m. with 10⁴ TCID₅₀ of the 
CSFV Shimen strain. Following challenge, the animals were monitored daily for the presence of clinical signs, including anorexia, depression, 
cough, dyspnea, and fever. At 14 dpc, all surviving pigs were euthanized. Various tissues from all the pigs were collected and subjected to pathological 
and IHC examinations.

**Blocking ELISA and serum-virus neutralizing test (SNT).** Serum 
samples at different time points were collected and tested for the produc-
tion of the gB-, gE-, and E2-specific antibodies by blocking ELISA using 
the PRV and CSFV antibody detection kits (Idexx, USA) according to the 
maker’s instructions.

The serum samples were also tested by SNT for the PRV- and CSFV-
specific serum neutralizing antibodies (NAbs) as described previously 
(17, 18). The titers of PRV- and CSFV-specific serum NAbs were deter-
dined and expressed as the reciprocal of the highest dilution at which 
infection of the PK-15 cells was inhibited in 50% of the culture wells.

**Virus isolation.** Nasal and rectal swabs were collected daily postim-
unization or postchallenge and subjected to virus isolation as described 
previously (17).

**Real-time RT-PCR.** Different tissues, including brain, spleen, lung, 
kidney, urinary bladder, tonsils, and lymph nodes, were collected at 14 
dpc and subjected to detection of the CSFV RNA by a real-time reverse transcription-PCR (RT-PCR) as described previously (21).

**Statistical analysis.** Data were analyzed using the SPSS 14.0 software. 
One-way analysis of variance (ANOVA) was performed using the 
Duncan’s multiple-
range test was used to compare the parameters among the different 
groups.

**RESULTS**

Generation of the recombinant virus rPRVTJ-delgE/gI-E2. Follow-
ing cotransfection with the pOK-LR-CMV-E2 plasmid and the 
digested genomic DNA of rPRVTJ-delgE/gI-EGFP, the recombinant 
virus was generated after six rounds of plaque purification. To obtain a recombinant virus strain with the highest ex-

FIG 3 Identification of rPRVTJ-delgE/gI-E2. (A) Western blotting of in-
fected PK-15 cells with rPRVTJ-delgE/gI-E2. (B) One-step growth curves of 
rPRVTJ-delgE/gI-E2. PK-15 cells were either infected with the CSFV Shimen strain or recombinant virus rPRVTJ-delgE/ 
gI-E2 or left uninfected for 48 h and then lysed for Western blotting using 
the anti-E2 MAb HQ06. The arrow indicates the band of the CSFV E2 
protein.
pression levels of the CSFV E2 protein and the propagation trait and with genetic stability, the recombinant virus was subjected to another round of purification by plaque assay and 10 plaque isolates were randomly picked out; after 20 passages on PK-15 cells, the inserted CSFV E2 gene of each plaque isolate was verified by PCR and sequencing (data not shown). The CSFV E2 protein was also detected in PK-15 cells infected with all plaque isolates of the recombinant virus by IFA (Fig. 2A).

PK-15 cells were infected with different plaque isolates of the recombinant virus at an MOI of 1. After a 12-h incubation, the infected cells were digested with trypsin and filtered with a 300-mesh sieve. After incubation with the anti-E2 MAb HQ06 and FITC-labeled goat anti-mouse IgG, respectively, the fluorescence intensity of protein expression was detected by flow cytometry. A plaque isolate, named rPRVTJ-delgE/gI-E2, with the highest expression of the E2 protein in infected PK-15 cells was screened (Fig. 2B). The E2 protein precipitated from lysates of rPRVTJ-delgE/gI-E2-infected PK-15 cells was similar in size to the native E2 protein precipitated from cells infected with CSFV (Fig. 3A). The one-step growth curve indicated that the in vitro growth of rPRVTJ-delgE/gI-E2 was comparable to that of rPRVTJ-delgE but different from that of the parent virus PRVTJ at the indicated time points. At 8 h postinfection (hpi), the titers of rPRVTJ-delgE and rPRVTJ-delgE/gI-E2 were only 10⁴ TCID₅₀/ml, in contrast with 10⁶ TCID₅₀/ml for the parent virus PRVTJ. There were significant differences between rPRVTJ-delgE, rPRVTJ-delgE/gI-E2, and PRVTJ (P < 0.05). Subsequently, the titers of each virus increased gradually and reached a peak at 14 to 18 hpi, with titers of 10⁸ (PRVTJ) and 10⁷ (rPRVTJ-delgE and rPRVTJ-delgE/gI-E2) TCID₅₀/ml (Fig. 3B).

Safety of rPRVTJ-delgE/gI-E2 for pigs. Following vaccination with rPRVTJ-delgE/gI-E2 or rPRVTJ-delgE, all animals remained clinically healthy and showed no adverse reactions. No fever was observed in any immunized pigs prior to challenge. In addition, no virus was detected in the nasal or rectal swabs of any inoculated animals prior to challenge.

FIG 4 Production of PRV-specific antibodies in immunized/challenged pigs. Groups of pigs (n = 5) were inoculated with different doses of rPRVTJ-delgE/gI-E2 or rPRVTJ-delgE or DMEM and challenged with the PRV TJ strain at the indicated time points. At 0, 3, and 6 days post-first immunization (dpi) and 0, 3, 6, 9, and 12 days postchallenge (dpc), serum samples were collected and tested for the presence of the anti-gB (A) and anti-gE (B) antibodies by using PRV antibody detection kits (Idexx, USA) according to the manufacturer’s instructions. ELISA values are given in S/N (sample OD₆₅₀ value/negative OD₆₅₀ value) ratios. Samples with S/N ratios less than 0.6 were scored positive. Standard deviations were shown as error bars.
TABLE 3 PRV-neutralizing antibodies in pigs following immunization with rPRVTJ-delgE/gl-E2 and challenge with PRV TJ strain

| Group   | Vaccine (TCID<sub>50</sub>) | Titer of NAb<sup>a</sup> at dpi (dpc): | 0   | 13 (6) | 16 (9) | 19 (12) |
|---------|-----------------------------|---------------------------------------|-----|--------|--------|--------|
| 1       | rPRVTJ-delgE/gl-E2 (10<sup>6</sup>) | <2                                   | <2  | 9.5 ± 1.8 | 22 ± 3.7 | 30 ± 3.7 |
| 2       | rPRVTJ-delgE/gl-E2 (10<sup>5</sup>) | <2                                   | <2  | 12.5 ± 3.2 | 14.5 ± 6.3 | 18 ± 4.8 |
| 3       | rPRVTJ-delgE/gI-E2 (10<sup>4</sup>) | <2                                   | <2  | 6 ± 1.8<sup>b</sup> | 10.7 ± 2.1<sup>b</sup> | 14 ± 3.1 |
| 4       | rPRVTJ-delgE (10<sup>3</sup>) | <2                                   | <2  | 14 ± 1.3   | 17.2 ± 4.1   | 20.8 ± 4.5 |
| 5       | DMEM                        | <2                                   | <2  | 2 ± 0       | 3.2 ± 1.0     | 5.6 ± 2.1   |

<sup>a</sup> There were significant differences between different doses of rPRVTJ-delgE/gl-E2 (10<sup>4</sup>, 10<sup>5</sup>, and 10<sup>6</sup> TCID<sub>50</sub>) at 6 and 9 dpc (P < 0.05), and there was no difference between groups immunized with 10<sup>3</sup> TCID<sub>50</sub> of rPRVTJ-delgE/gl-E2 and rPRVTJ-delgE at any indicated time points.

<sup>b</sup> The titers of PRV-specific serum neutralizing antibodies (NAb) are expressed as the reciprocal of the highest dilution at which infection of the PK-15 cells was inhibited in 50% of the culture wells.

**PRV-specific antibodies induced by rPRVTJ-delgE/gl-E2 in pigs.** At 6 days postimmunization (dpi), the gB-specific antibodies were detected in pigs immunized with rPRVTJ-delgE/gl-E2 and rPRVTJ-delgE. Following challenge with the PRV variant TJ strain, the gB-specific antibodies increased progressively and reached a peak at 9 dpc (Fig. 4A). The gE-specific antibodies were not detected in all pigs immunized with rPRVTJ-delgE/gl-E2 or rPRVTJ-delgE before 12 dpc but were detected in the DMEM group (3/5) or in the pigs immunized with 10<sup>4</sup> TCID<sub>50</sub> of rPRVTJ-delgE/gl-E2 (3/5) at 12 dpc (Fig. 4B).

The SNT results showed that anti-PRV NAb were not detectable in all groups until 6 dpc. At 6, 9, and 12 dpc, the anti-PRV NAb were detected in groups immunized with rPRVTJ-delgE/gl-E2 at different doses or with rPRVTJ-delgE. There were significant differences between different doses of rPRVTJ-delgE/gl-E2 (10<sup>4</sup>, 10<sup>5</sup>, and 10<sup>6</sup> TCID<sub>50</sub>) (P < 0.05), with no difference between groups immunized with 10<sup>5</sup> TCID<sub>50</sub> of rPRVTJ-delgE/gl-E2 and rPRVTJ-delgE at any indicated time points (Table 3).

**Protection of pigs immunized with rPRVTJ-delgE/gl-E2 from virulent PRV challenge.** No clinical signs typical of PR were observed in pigs immunized with rPRVTJ-delgE/gl-E2 and rPRVTJ-delgE after the virulent PRV TJ strain challenge. In the DMEM group, all pigs displayed typical PR signs (depression, anorexia, cough, diarrhea, and neurological signs) with high fever (>40.5°C) from 1 dpc to the end of the PRV challenge experiment. The challenge virus was isolated from the nasal and rectal swabs of all pigs in the DMEM group (5/5) and one pig in the group immunized with 10<sup>5</sup> TCID<sub>50</sub> of rPRVTJ-delgE/gl-E2, whereas no challenge virus was isolated in other groups (Table 4).

**CSFV-specific antibodies induced by rPRVTJ-delgE/gl-E2 in pigs.** E2-specific antibodies were measured by blocking ELISA and SNT following vaccination. Four weeks after the first immunization, E2-specific antibodies were detectable in two pigs immunized once with high-dose (10<sup>5</sup>-TCID<sub>50</sub>) rPRVTJ-delgE/gl-E2 and two pigs immunized twice with low-dose (10<sup>4</sup>- or 10<sup>5</sup>-TCID<sub>50</sub>) rPRVTJ-delgE/gl-E2, with the antibody titers increasing gradually. After virulent CSFV challenge, the antibody titers in pigs immunized with rPRVTJ-delgE/gl-E2 or the C-strain were increased markedly. There were significant differences in antibody titers between the C-strain and other groups and between the double-shot groups immunized with 10<sup>5</sup> TCID<sub>50</sub> or 10<sup>4</sup> TCID<sub>50</sub> of rPRVTJ-delgE/gl-E2, and the one-shot rPRVTJ-delgE/gl-E2 group (10<sup>6</sup> TCID<sub>50</sub>) at 28, 35, and 38 dpi (P < 0.05), but there were no significant differences in antibody titers between the C-strain group and groups immunized with different doses of rPRVTJ-delgE/gl-E2 after 9 dpc. None of the pigs in the PBS group produced detectable CSFV-specific antibodies throughout the experiment (Fig. 5).

Anti-CSFV NAb were first detected in double-shot rPRVTJ-delgE/gl-E2 (10<sup>5</sup>- or 10<sup>6</sup>-TCID<sub>50</sub>) groups at 28 dpi and the one-shot rPRVTJ-delgE/gl-E2 group (10<sup>6</sup> TCID<sub>50</sub>) at 28, 35, and 38 dpi (P < 0.05), but there were no significant differences in antibody titers between the C-strain group and other groups immunized with different doses of rPRVTJ-delgE/gl-E2 after 9 dpc. None of the pigs in the PBS group produced detectable CSFV-specific antibodies throughout the experiment (Fig. 5).

**Protection of pigs immunized with rPRVTJ-delgE/gl-E2 from lethal CSFV challenge.** No clinical signs were observed following challenge in all groups, except the PBS group. In the PBS group, all pigs displayed typical CSF signs (anorexia, depression, chill, prostration, incoordination, and constipation followed by diarrhea, locomotor ataxia, and posterior paresis) with high fever (>40.5°C) from 2 dpc. All the pigs in the PBS group died from 6 to 41 dpc (Table 5).

**Protection of pigs immunized with rPRVTJ-delgE/gl-E2 from lethal CSFV challenge.** No clinical signs were observed following challenge in all groups, except the PBS group. In the PBS group, all pigs displayed typical CSF signs (anorexia, depression, chill, prostration, incoordination, and constipation followed by diarrhea, locomotor ataxia, and posterior paresis) with high fever (>40.5°C) from 2 dpc. All the pigs in the PBS group died from 6 to 41 dpc (Table 5).

**Table 4 Outcomes for immunized pigs following virulent PRV TJ strain challenge**

| Group   | Vaccine          | Dose (TCID<sub>50</sub> unless otherwise specified) | No. of days to fever onset | Fever incidence<sup>a</sup> | Survival rate (no. of pigs surviving/total no. of pigs) | Viral shedding (no. of pigs shedding/total no. of pigs) |
|---------|------------------|-----------------------------------------------------|----------------------------|-----------------------------|--------------------------------------------------------|-------------------------------------------------------|
| 1       | rPRVTJ-delgE/gl-E2 | 10<sup>6</sup>                                       | 0/75                       | 5/5                         | 0/5                                                    | 0/5                                                   |
| 2       | rPRVTJ-delgE/gl-E2 | 10<sup>5</sup>                                       | 0/75                       | 5/5                         | 0/5                                                    | 0/5                                                   |
| 3       | rPRVTJ-delgE/gl-E2 | 10<sup>5</sup>                                       | 0/75                       | 5/5                         | 1/5                                                    | 1/5                                                   |
| 4       | rPRVTJ-delgE     | 10<sup>5</sup>                                       | 0/75                       | 5/5                         | 0/5                                                    | 0/5                                                   |
| 5       | DMEM             | 1 ml                                                | 1                          | 21/36                       | 1/5                                                    | 5/5                                                   |

<sup>a</sup> Total no. of days with any pig showing fever (>40.5°C)/total days of monitoring for all pigs in a group following virulent challenge.
At 14 dpi, all surviving pigs were euthanized and subjected to pathological examination. None of the pigs immunized with $10^5$ or $10^6$ TCID$_{50}$ of rPRVTJ-delgE/gI-E2 or the C-strain showed pathological changes. Some (2/5) pigs immunized with $10^4$ TCID$_{50}$ of rPRVTJ-delgE/gI-E2 showed mild lesions (including slight hemorrhages in the lymph nodes, bladder, heart, and spleen). The pigs in the PBS group all showed severe pathological changes, including hemorrhages with necrotic foci in the tonsils, enlargement and hemorrhages of the lymph nodes, infarcts in the spleen, extensive petechiae in the kidney and bladder, and button-like ulcers in the ileocecal valve (data not shown). Examination of brain, spleen, lungs, kidney, bladder, tonsils, or lymph nodes by real-time RT-PCR showed that no CSFV RNA was detectable in these tissues of any animal of the vaccination groups 1, 2, 3, and 6 at 14 dpc. As expected, the tissues of all nonvaccinated control animals (group 7) contained between $7.2 \times 10^4$ and $6.3 \times 10^5$ CSFV RNA copies (data not shown).

**DISCUSSION**

Vaccination represents one of the most effective prophylactic measures to protect pigs against viral infections. Among the various types of vaccines available, MLVs are often preferred over inactivated or subunit ones, because MLVs are able to induce long-lasting humoral and cell-mediated immunity (22).

Despite tremendous efforts invested in controlling PR and CSF, the diseases continue to plague the swine industry in many countries. It has been demonstrated that pigs are occasionally coinfected with PRV and CSFV or other viruses, such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), or porcine parvovirus (PPV), in the field in China (23), and coinfections usually cause more severe wasting diseases. So, using PRV as a vector to develop bivalent or multivalent vaccines will be of great significance.

Recently, we constructed a gE/gI-gene-deletion PRV mutant, rPRVTJ-delgE, which is defective for the gE and gI genes. We showed that rPRVTJ-delgE was safe and immunogenic for pigs and was able to protect immunized pigs from lethal PRV challenge (17). This suggests that rPRVTJ-delgE may be used as a biologically safe vaccine vector for the expression of other viral antigens. To verify this, we inserted the gene encoding the envelope glycoprotein E2 of CSFV into the genome of rPRVTJ-delgE under the transcriptional control of the human cytomegalovirus (hCMV)

**TABLE 5 CSFV-specific neutralizing antibodies in pigs following immunization and challenge with lethal CSFV**

| Group | Vaccine (TCID$_{50}$) | Antibody titer at dpi (dpc): |
|-------|-----------------------|-----------------------------|
|       |                       | 0  | 28 | 35 (0) | 38 (3) | 41 (6) | 44 (9) | 47 (12) |
| 1     | rPRVTJ-delgE/gI-E2 ($10^6$) | <8 | <8 | 32 ± 12 | 56 ± 25 | 108 ± 56 | 384 ± 136 | 512 ± 0 |
| 2     | rPRVTJ-delgE/gI-E2 ($10^5$) | <8 | 20 ± 12 | 40 ± 15 | 60 ± 22 | 80 ± 30 | 384 ± 167 | 480 ± 59 |
| 3     | rPRVTJ-delgE/gI-E2 ($10^4$) | <8 | 26 ± 28 | 42 ± 16 | 53 ± 8 | 74 ± 16 | 426 ± 132 | 512 ± 0 |
| 6     | C-strain              | <8 | 47 ± 11$^a$ | 67 ± 9$^a$ | 112 ± 23$^b$ | 336 ± 29$^b$ | 512 ± 0 | 512 ± 0 |
| 7     | PBS                   | <8 | <8 | <8 | <8 | 10 ± 4 | 29 ± 27 |

$^a$ There were significant differences between the C-strain group and other groups at 28 and 35 dpi ($P < 0.05$).

$^b$ There were very significant differences between the C-strain group and other groups at 38 and 41 dpi ($P < 0.001$).

$^c$ The titers of CSFV-specific serum neutralizing antibodies (NABs) are expressed as the reciprocal of the highest dilution at which infection of the PK-15 cells was inhibited in 50% of the culture wells.
immediate-early promoter. It has been shown that E2 is highly immunogenic and can provide protection for pigs against CSF (18, 24).

To develop a virus vector-based bivalent vaccine, it is important that the recombinant virus still retains the growth ability and immunogenicity of the vector virus. In this study, we demonstrated that there was no difference in virus titers between rPRVTJ-delgE/gl-E2 and the parent virus rPRVTJ-delgE, indicating that the insertion of the E2 gene in the ge/gl locus did not influence the growth of the vector virus.

Following vaccination with rPRVTJ-delgE/gl-E2, all animals remained clinically healthy and showed no adverse reactions, and no virus shedding was detected in the nasal or rectal swabs of all inoculated animals. In a recent study, we demonstrated that a maximum amount up to 10⁴ TCID₅₀/ml of PRV shedding could be detected in the pigs infected with PRVTJ at 4 to 6 dpc (unpublished data).

Pigs immunized with different doses of rPRVTJ-delgE/gl-E2 induced strong PRV-specific humoral immune responses, which are dose dependent; rPRVTJ-delgE/gl-E2 and rPRVTJ-delgE immunizations at the same dose induced comparable levels of PRV-specific antibodies (Table 3) and full protection from lethal PRV challenge, which indicates that the insertion of a foreign gene did not influence the immunogenicity of the vector virus.

Several CSF marker vaccines, such as pSFV1CS-E2 (25), pcdSW (26), or rAdV-E2 (18), have been shown to provide incomplete protection from lethal CSFV challenge. In contrast, rPRVTJ-delgE/gl-E2 showed a much greater efficacy, since it was able to provide complete protection even when administered as a single dose, which was comparable to that of rAdV-SFV-E2 (24) or CP7_E2aII (27).

In order to determine the dose that yields full protection against PRV and CSFV challenge, pigs were immunized with three different titers of rPRVTJ-delgE/gl-E2, and all pigs vaccinated with different doses of rPRVTJ-delgE/gl-E2 showed full protection in PRV and CSFV challenge experiments. The minimum dose of rPRVTJ-delgE/gl-E2 protective against PR is expected to be lower than 10⁴ TCID₅₀. In subsequent experiments, we will evaluate the immune efficacy of lower doses.

In this study, a rapid increase in CSFV- or PRV-specific neutralizing antibody titers was observed in pigs immunized with different doses of rPRVTJ-delgE/gl-E2 following virulent challenge, indicating that immunization with rPRVTJ-delgE/gl-E2 could establish an immunological memory. In pigs, rPRVTJ-delgE/gl-E2 was safe even when used for inoculation at 10⁶ TCID₅₀. Thus, the recombinant virus rPRVTJ-delgE/gl-E2 has a potential to be developed as a bivalent vaccine.

Nevertheless, the present data demonstrated the efficacy of the recombinant virus only in piglets. Prior to practical application, it is necessary to further study whether rPRVTJ-delgE/gl-E2 can protect pregnant sows against reproductive failure and confer sterilizing immunity in sows and their offspring. It is also necessary to further study this vaccine candidate using different immunization methods, such as intradermal and parenteral routes. In addition, the latency of this new recombinant should be evaluated.

In summary, we describe here a recombinant PRV expressing the CSFV E2 protein vectored by a ge/gl-deleted PRV variant with high safety and strong immunogenicity in pigs. This recombinant virus might be a promising bivalent vaccine candidate against PRV and CSFV coinfections of pigs.

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Table 6: Outcomes for immunized pigs following virulent CSFV challenge

| Group | Vaccine               | Dose (TCID₅₀ unless otherwise specified) | No. of days to fever onset | Fever incidence* | Survival rate (no. of pigs surviving/total no. of pigs) |
|-------|-----------------------|-----------------------------------------|----------------------------|------------------|----------------------------------------------------------|
| 1     | rPRVTJ-delgE/gl-E2    | 10⁶                                     | 0/75                       | 5/5              |                                                          |
| 2     | rPRVTJ-delgE/gl-E2    | 10⁶                                     | 0/75                       | 5/5              |                                                          |
| 3     | rPRVTJ-delgE/gl-E2    | 10⁶                                     | 0/75                       | 5/5              |                                                          |
| 6     | C-strain              | One dose                                | 2                          | 17/35            | 5/5                                                      |
| 7     | PBS                   | 1 ml                                    |                             | 0/5              |                                                          |

*Total number of days with any pig showing fever (≥40.5°C)/total number of days of monitoring for all pigs in a group following virulent challenge.
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