A novel gE-deleted pseudorabies virus (PRV) provides rapid and complete protection from lethal challenge with the PRV variant emerging in Bartha-K61-vaccinated swine population in China

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A B S T R A C T

The currently used Bartha-K61 strain is a very safe and effective vaccine against pseudorabies (PR) and has played a critical role in the control and eradication of PR worldwide. Since late 2011, however, PR reemerged among Bartha-K61-vaccinated pig population in many regions in China. Our previous studies demonstrated that the Bartha-K61 vaccine was unable to provide complete protection from the challenge with the PRV TJ strain (PRVTJ), a representative emerging PRV variant that was isolated from a Bartha-K61-immunized pig farm in Tianjin, China. Here, we generated a gE-deleted PRV, named as rPRVTJ-delgE, based on PRVTJ and evaluated its safety and immunogenicity in pigs. Our results showed that groups of piglets (n = 5) immunized with 10^1, 10^4 or 10^5 TCID50 rPRVTJ-delgE did not exhibit clinical signs following immunization and challenge and were protected clinically and virologically from the lethal challenge with PRVTJ as early as 1 week post-immunization, in contrast with the incomplete protection provided by the Bartha-K61 vaccine. These indicate that rPRVTJ-delgE is a promising candidate vaccine for updating Bartha-K61 for the control of the currently epidemic PR in China.

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1. Introduction

Pseudorabies (PR) or Aujeszky’s disease (AD), caused by pseudorabies virus (PRV), is a serious disease of pigs and other animals, leading to significant economic losses to the pig industry in many countries. The disease is characterized by neurological signs, severe respiratory illness, abortions, reduced litter size and decreased growth rates of survivors. The disease is associated with high morbidity and mortality in newborn piglets [1–3].

The currently used Bartha-K61 strain is a very safe and effective vaccine against PR and has played a critical role in the control and eradication of PR. Since late 2011, however, PR has occurred among Bartha-K61-vaccinated pigs on many farms in China. We isolated and characterized a PRV variant (named as PRVTJ) from brain samples from a pig farm in Tianjin, China. Sequence analysis indicated that PRVTJ is closely related to other recently emergent PRV variants isolated by different groups in China. These PRV variants were all clustered to a relatively independent clade in the phylogenetic tree [4–6,16]. Compared with the classical PRV strains such as Kaplan and Becker strains, the gE gene of PRVTJ contains 2 amino acid (aa) (D) insertions at positions 48 and 494 and 18 aa point mutations (Fig. S2); the gI gene of PRVTJ contains 1 aa (H) deletion at position 172, and 1 aa (G) insertion at position 238, and 17 point mutations (Fig. S2); the gB gene of PRVTJ contains 3 aa (S, P and G) deletions at positions 75–77, 1 aa (G) insertion at position 94 and 22 point mutations (Fig. S4); and the gD gene of PRVTJ contains 2 aa (R and P) insertions at positions 280 and 281 (Fig. S5). The experimental challenge infection results indicated that pigs immunized with the Bartha-K61 vaccine were not completely protected from the challenge with PRVTJ. The vaccine-immunized pigs exhibited moderate PR clinical signs and pathological changes, and unvaccinated pigs showed severe PR clinical signs and pathological changes and some died of challenge infection upon the PRVTJ challenge. Notably, some non-vaccinated, challenged pigs exhibited deaths and pruritus, which were rarely found in previous PRV infections (unpublished data).

Vaccination has been an integral part of eradication programs for PRV. TK, gE or/and gE-gene-deleted PRV vaccines are safe and
effective against PRV infection [1,7,8]. The PRV Bartha-K61 strain is a widely used modified live vaccine with the gE/gI deletion. The gE is dispensable for viral replication but is essential for transneuronal spread of PRV [9,10]. Deletion of the gE gene results in marked attenuation of the virus in almost all animal species that are susceptible to PRV, because the gE-deleted PRV has a reduced capability to infect second- and third-order neurons of the olfactory and trigeminal routes in the central nervous system [11,12]. Thus gE is a major virulence determinant of PRV.

Referring to the deletion of PRV Bartha-K61 strain, we generated a gE-deleted PRV mutant derived from the variant PRVTJ and evaluated its safety and immunogenicity in pigs.

2. Materials and methods

2.1. Viruses and cells

The PRV TJ strain (PRVTJ) was isolated from the brain samples of diseased pigs suspected of PR from Bartha-K61-vaccinated farm in Tianjin, China in 2012. The virus has been adapted to and plaque-purified in PK-15 cells (ATCC CCL-33). PRV Bartha-K61 strain vaccine (lot number 2012002) was produced by Weike Biotech Co., Harbin, China. PK-15 and Vero cells were grown 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 100IU/ml penicillin at 37 °C in a humidified 5% CO₂ atmosphere.

2.2. Construction of transfer vectors

The PRV transfer vector left and right homologous arms (flanking the PRV gE and gI genes, named as L and R) were amplified by PCR using primer pairs P1S/P1R and P2S/P2R (Table S1). The resulting L and R fragments joined together with the MluI site were ligated into the EcoRI and Xhol sites of the pOK12 vector (Novagen, USA), resulting in pOK-LR (Fig. S1). The EGFP expression cassette (under the control of CMV promoter and polyA terminator) was amplified with primers P3S and P3R (Table S1) from pEGFP-N1 (Clontech, USA) and cloned into the MluI site of pOK-LR, creating pOK-LR-EGFP (Fig. S1).

2.3. Generation of virus mutants

The PRVTJ virions were concentrated by ultracentrifugation and the viral genomic DNA was extracted using the DNA Tissue Kit (Omega, USA) according to the manufacturer’s instructions. Vero cells (2 × 10⁵ cells/well) were co-transfected with the plasmid pOK-LR-EGFP and the genomic DNA of PRVTJ using the X-tremeGENE HP DNA transfection reagent (Roche, USA) according to the manufacturer’s instructions. After development of cytopathic effects (CPEs), cell monolayers were overlaid with 1% -containing 2% FBS in DMEM for plaque purification based on the EGFP expression. Plaques showing green fluorescence under fluorescent microscopy were purified after multiple rounds of screening in PK-15 cells. The presence of the EGFP gene and the gE gene and the absence of the gE gene were verified by PCR using EGFP-specific (P7S/P7R), gE-specific (P6S/P6R) and gI-specific (P5S/P5R) primer pairs (Table S1). The expected gE/gI-deleted virus expressing EGFP was named as rPRVTJ-delgE-EGFP.

The gE/gI-deleted, EGFP-free PRV mutant rPRVTJ-delgE was generated in Vero cells co-transfected with the plasmid pOK-LR and the PmlI-digested genomic DNA of rPRVTJ-delgE-EGFP, as described above. The absence of the PRV gE gene was verified by PCR using P4S/P4R or P5S/P5R primer pairs (Table S1).

2.4. Plaque assays

The PK-15 cell monolayers in six-well culture plates were infected with serially diluted rPRVTJ-delgE-EGFP or rPRVTJ-delgE. After 1-h incubation, the medium was aspirated, and the cells were overlaid with 2 ml of 1% low-melting point agarose in DMEM. After solidification of the agarose, plates were incubated at 37 °C in a 5% CO₂ atmosphere for 16–20 h for plaque development. Fluorescent plaque visualization was performed under a fluorescent imaging system. Moreover, the PK-15 cell monolayers in six-well culture plates were infected with PRVTJ or rPRVTJ-delgE at the same multiplicity of infection (MOI) for plaque comparisons as described above.

2.5. Immunofluorescence assay (IFA)

To check the expression of the gE protein, PK-15 cells were infected with rPRVTJ-delgE or PRVTJ at an MOI of 1 for 24 h. Cells were fixed with cold acetone for 10 min. The fixed cells were incubated with the mouse anti-gE or anti-gB monoclonal antibody (mAb) (1:100 dilution, IDEXX, USA) for 2 h at 37 °C in a humidified chamber followed by three washes with PBS. The cells were then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma-Aldrich, USA) for 1 h at 37 °C, followed by three washes with PBS. The cells were covered with 90% alkaline glycerine and examined under fluorescence microscope (Nikon TE200, Japan).

2.6. One-step growth curve

One-step growth curves were performed for Bartha-K61, rPRVTJ-delgE, rPRVTJ-delgE-EGFP and PRVTJ as described previously [13]. Briefly, PK-15 cells grown in 24-well culture plates were inoculated at 1 MOI with each of the above viruses. After 1-h incubation at 37 °C in a 5% CO₂ atmosphere, the inoculum was aspirated, and cells were washed three times with PBS to remove the unabsorbed virus and incubated with fresh DMEM. The culture supernatants were collected at the indicated time points (Fig. 1B), and used to determine the viral titers. Growth curves for each virus tested were performed in duplicates, and the resulting titers were averaged.

2.7. Immunogenicity test of rPRVTJ-delgE in pigs

Twenty-one 6-week-old healthy piglets from a PRV-free swine herd were randomly allocated into A, B, C, D and E groups. Pigs in Group A (n = 3) were inoculated intramuscularly (i.m.) with one-dose (10⁵ TCID₅₀) of PRV Bartha-K61 strain vaccine. Groups B, C and D (n = 5) were immunized i.m. with 10², 10⁴ and 10⁵ TCID₅₀ rPRVTJ-delgE, respectively. Pigs in group E were injected i.m. with PBS as control. One week post-immunization, all pigs were challenged i.m. with 10⁵ TCID₅₀ PRVTJ in the neck. After challenge, clinical signs and rectal temperatures were monitored daily throughout the experiment. The animal experiment was approved by the Institutional Animal Care and Use Committee and conventional animal welfare regulations and standards were taken into account.

2.8. Blocking enzyme-linked immunosorbent assay (ELISA) and serum-virus neutralizing test (SN)

At 0, 3, 6 days post-immunization (DPI) and 0, 3, 6, 9, 12 days post-challenge (DPC) serum samples were collected and tested for the presence of the gB- and gE-specific antibodies by using the PRV antibody detection kits (IDEXX, USA) according to the manufacturer’s instructions.
Fig. 1. Identification of the recombinant viruses. (A) The gE and gB protein expression identified by IFA. (B) One-step growth curves of the recombinant viruses. PK-15 cells grown in a 24-well plate were inoculated at 1 MOI with Bartha-K61, rPRVTJ-delgE-EGFP, rPRVTJ-delgE or PRVTJ. The culture supernatants were collected at the indicated time points, and used to determine the viral titers. Error bars represent the standard errors of the mean from two replicates. *, significant difference; **, very significant difference.

Serum samples were also tested by SNT for the PRV specific neutralizing antibodies (NAb) [14,15]. Briefly, sera were diluted twofold serially and mixed with 100 TCID<sub>50</sub> PRVTJ and incubated at 37°C for 1 h. The serum–virus mixture was inoculated into the confluent PK-15 cells cultured in 96-well plates and incubated at 37°C for 1 h. The inoculated cells were then incubated at 37°C for 48–72 h for development of CPEs to determine the titers of PRV-specific NAb, and the titers were expressed as the reciprocal of the highest dilution at which infection of the PK-15 cells was inhibited in 50% of the culture wells.

2.9. Pathology and immunohistochemistry (IHC)

At 14 DPC, all surviving pigs were euthanized and organs (brain, lung, liver, kidney, heart, spleen, bladder, tonsils and lymph nodes)
collected and subjected to pathological and IHC examinations as described previously [16].

2.10. Virus isolation

Nasal and rectal swabs were collected daily post-immunization and post-challenge, and different tissues including brain, heart, liver, spleen, lung, kidney, urinary bladder, tonsils and lymph nodes collected at 14 DPC and subjected to virus isolation as described previously [17]. Briefly, tissue homogenates were prepared and centrifuged at 3000 rpm for 10 min. The supernatant was passed through a 0.45-μm filter and inoculated into Vero cell monolayers followed by three blind passages. After development of CPEs, the virus DNA was extracted using the DNA Tissue Kit (Omega, USA) according to the manufacturer’s instructions, and subjected to PCR using gE- and gB-specific primers (Table S1).

2.11. PCR

The total DNA was extracted from different tissues of the challenged pigs by using the DNA Tissue Kit (Omega, USA), and the presence of the challenge virus genomic DNA was examined by PCR using the gE-specific primers PSS and P5R (Table S1).

2.12. Statistical analysis

Data were analyzed using the SPSS 14.0 software. One-way ANOVA followed by Duncan’s multiple range test was used to compare the parameters among the different groups.

3. Results

3.1. Characterization of the recombinant viruses

Plaques with green fluorescence were produced by cotransfection with the pOK-LR-EGFP plasmid and the genomic DNA of PRVTJ and screened by plaque purification to generate the gE-deleted, EGFP-expressing virus rPRVTJ-delgE-EGFP. After cotransfection with the pOK-LR plasmid and the genomic DNA of rPRVTJ-delgE-EGFP, the plaques without green fluorescence were screened and purified for ten rounds, resulting in the gE-deleted, EGFP-negative virus rPRVTJ-delgE. PCR identification showed that the gB gene, but not the EGFP and gE genes, was detectable from the genome of rPRVTJ-delgE. Furthermore, the plaque formation of rPRVTJ-delgE was slower than that of PRVTJ in spite of similar plaque sizes (data not shown). The gB protein was detected by IFA in PK-15 cells infected with PRVTJ or rPRVTJ-delgE, whereas the gE protein was only detected in PK-15 cells infected with PRVTJ (Fig. 1A). One-step growth curves indicated that the in vitro growth of the gE-deleted mutants was attenuated compared to the parent virus PRVTJ (p < 0.001) (Fig. 1B).

3.2. Safety of rPRVTJ-delgE for pigs

Following vaccination with rPRVTJ-delgE or Bartha-K61, all animals remained clinically healthy and showed no adverse reactions. No elevation of rectal temperatures was observed in all pigs before challenge. In addition, rPRVTJ-delgE or Bartha-K61 was not detectable in nasal and rectal swabs of all inoculated animals prior to challenge.

3.3. Antibodies induced by rPRVTJ-delgE in pigs

At 6 DPI, the gB-specific antibodies were detected in pigs immunized with rPRVTJ-delgE or Bartha-K61. After challenge, the gB-specific antibodies increased progressively, and reached a peak at 9 DPC (Fig. 2A). The gE-specific antibodies were not detected in all pigs before 9 DPC (Fig. 2B), but detected in some Bartha-K61-immunized pigs (2/3) and 10(2.83 ± 0.14) TCID50 rPRVTJ-delgE-immunized pigs (1/5) at 12 DPC.

Anti-PRV NAb s were examined at 0, 3, and 6 DPI and 0, 3, 6, and 9 DPC. The results showed that anti-PRV NABs were not detected in all groups except the 10(3 TCID50 rPRVTJ-delgE-immunized pigs (2.5 ± 0.58) before challenge. At 3 DPC, the average NABs were detectable in all immunized groups: with the highest titer in 10(5 TCID50 rPRVTJ-delgE (7.75 ± 1.75), followed by 10(4 TCID50 rPRVTJ-delgE (6.25 ± 1.67), 10(3 TCID50 rPRVTJ-delgE (5.25 ± 0.53) and Bartha-K61 (5.00 ± 0.00), and there was significant difference between the 10(3 TCID50 rPRVTJ-delgE group and the Bartha-K61 group (p < 0.05). At 6 DPC, the anti-PRV NABs in the vaccinated groups increased further with the highest titer in the 10(5 TCID50 rPRVTJ-delgE group, and there was very significant difference between the 10(3 TCID50 rPRVTJ-delgE group and the Bartha-K61 group (p < 0.001) (Table 1). No neutralizing titers were detected in the unvaccinated group throughout the experiment.

3.4. Protection of pigs immunized with rPRVTJ-delgE from virulent challenge

No clinical signs typical of PR were observed in pigs immunized with different doses of rPRVTJ-delgE after virulent challenge. Two out of three pigs in the Bartha-K61 vaccine group exhibited fever, depression, anorexia and retarded growth from 4 DPC. In the PBS group, all pigs displayed typical PR signs (depression, anorexia, cough, diarrhea and systemic neurological signs) with high fever (40.5–42 °C) from 1 DPC till death. The fever frequencies were the highest for the unvaccinated group (15/42), followed by the Bartha-K61 vaccine group (3/42) (Table 2). Two pigs in the PBS group died at 5 DPC, while the pigs in other groups all survived. The challenge virus was isolated from the nasal and rectal swabs of three unvaccinated pigs (with the shedding virus titers of 10(2.83 ± 0.14) TCID50/ml at 2–14 DPC and two pigs immunized with the Bartha-K61 vaccine (with the titers of 10(1.16 ± 0.18) TCID50/ml) at 4–5 DPC. There was a significant difference between these two groups (p = 0.01). In contrast, no challenge virus shedding was detected in all pigs vaccinated with different doses of rPRVTJ-delgE.

All dead and surviving pigs were subjected to pathological examination. Pigs in the PBS group showed severe pathological changes, including obvious hemorrhages in the brain and lymph nodes. Two pigs in the Bartha-K61 vaccine group showed mild lesions (such as slight hemorrhages in the brain, lymph nodes and lung). None of the pigs immunized with rPRVTJ-delgE displayed pathological changes. The histopathologic changes of unvaccinated pigs were characterized by local hemorrhages in the meninges, meninitis, edema and hemorrhage in the lungs, foci of yellow-white necrosis in the kidneys and lymphocyte infiltration around the small blood vessels of the brain cortex (data not shown). The challenge virus DNA was detected by PCR in various organs of the unvaccinated pigs and in the tonsils of one pig immunized with Bartha-K61, but not in any tissues of the pigs immunized with rPRVTJ-delgE.

4. Discussion

Since late 2011, outbreaks of PR occurred in many vaccinated pig farms in China and caused great economic losses to the swine industry. The disease is characterized by high fever, depression, respiratory distress, anorexia, cough, shivering, diarrhea, and stillbirth or the birth of weak piglets with neurologic signs that ultimately lead to death. The onset of clinical signs in newborn piglets is sudden, spanning about 5 h from onset to death. The
Fig. 2. Production of PRV-specific antibodies in immunized/challenged pigs. At 0, 3, and 6 days post-immunization (DPI) and 0, 3, 6, 9, and 12 days post-challenge (DPC), serum samples were collected and tested for the presence of the anti-gB (A) and anti-gE (B) antibodies by using the PRV antibody detection kits (IDEXX, USA) according to the manufacturer’s instructions.

Table 1

Neutralizing antibodies in pigs following immunization and challenge.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days post-immunization (DPI)</th>
<th>Days post-challenge (DPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Bartha-K61</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>rPRVTJ-delgE 10^5 TCID_{50}</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>rPRVTJ-delgE 10^4 TCID_{50}</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>rPRVTJ-delgE 10^6 TCID_{50}</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

Serum samples were tested for the PRV-specific neutralizing antibodies (NAbs). Briefly, sera were diluted twofold serially and mixed with 100 TCID_{50} PRVTJ and incubated at 37°C for 1 h. The serum-virus mixture was inoculated into the confluent PK-15 cells cultured in 96-well plates and incubated at 37°C for 1 h. The inoculated cells were then incubated at 37°C for 48–72 h for development of CPEs to determine the titers of PRV-specific NAbs, and the titers were expressed as the reciprocal of the highest dilution at which infection of the PK-15 cells was inhibited in 50% of the culture wells.

* Significant difference between the rPRVTJ-delgE (10^5 TCID_{50}) group and the Bartha-K61 group (p < 0.05)

** Very significant difference between rPRVTJ-delgE (10^5 TCID_{50}) group and the Bartha-K61 group (p < 0.001).
mortality of infected neonatal piglets is up to 50% [4–6,18]. It has been demonstrated that the Bartha-K61 vaccine was effective against the lethal challenge with the classical PRV SC strain, but did not provide full protection against the emerging PRV variant [4], which indicates that the emerging PRV is immunogenically different from Bartha-K61. The disease is spreading quickly and the swine population in China is under the great threat of the epidemic.

The PRV variant PRV\textsubscript{TV} was isolated from a Bartha-K61-vaccinated pig farm in Tianjin, China in 2012. According to the Quality Standards for Veterinary Biologic Products in China, we determined the efficacy of the Bartha-K61 vaccine against PRV\textsubscript{TV} in sheep model, and showed that Bartha-K61-immunized sheep exhibited typical PR clinical signs as early as 1 DPC with PRV\textsubscript{TV} and mostly died at 4–5 DPC. These indicate that Bartha-K61 cannot provide complete protection against the emerging PRV\textsubscript{TV}, urging the need for updating of the currently used PR vaccines [4].

Attenuated live vaccines and gene-deleted mutant vaccines accompanied with corresponding differential diagnostic tests have played an important role in the control and eventual eradication of PR [19]. It has been identified that the gE and gI genes are related to the virulence, but not essential for the immunogenicity of PRV [20–23]. According to the gene-deletion region of the Bartha-K61 strain, we generated a gE-deleted mutant vaccine rPRV\textsubscript{TV}-delgE based on the variant PRV\textsubscript{TV}. PCR, IFA and sequencing indicated that the gE gene was deleted as expected. The one-step growth curves showed that the titers of Bartha-K61, rPRV\textsubscript{TV}-delgE and rPRV\textsubscript{TV}-delgE-EGFP were slightly lower than that of PRV\textsubscript{TV}, which demonstrates a clear progression of attenuated growth kinetics (p < 0.001) (Fig. 1B).

Following vaccination with rPRV\textsubscript{TV}-delgE at different doses, all animals remained clinically healthy and showed no adverse reactions and elevation of rectal temperatures. The rPRV\textsubscript{TV}-delgE virus was not detectable in the nasal and rectal swabs of all immunized animals prior to challenge. These indicate that the gE/gI gene deletion resulted in the reduced virulence of PRV\textsubscript{TV}, which is consistent with previous findings that gE and gI genes are critical virulence factors for PRV [20,21,24]. Prior to challenge, gE-specific ELISA antibodies, but not PRV\textsubscript{TV}-specific NAbs, were detected in rPRV\textsubscript{TV}-delgE- or Bartha-K61-immunized pigs, which were completely or partially protected from the virulent challenge. This implies that protection against the PRV challenge does not necessarily correlate with the antibody titers. At 9 DPC, gE-specific ELISA antibodies were detected in some pigs (2/3) immunized with Bartha-K61, indicating that the replication of the challenge virus occurred due to the incomplete protection provided by the Bartha-K61 vaccine. In this study, only two out of three Bartha-K61-vaccinated pigs displayed fever and obvious PR clinical signs, two-day virus shedding, without mortality. The mortality is not as high as that observed in neonates in the field. The possible explanation is that pigs used in this study are much older (about 8-week-old at challenge time). Next we will test if younger piglets immunized Bartha-K61 will survive in the challenge with the PRV variant.

It is noteworthy that rPRV\textsubscript{TV}-delgE-immunized pigs were completely protected from the lethal challenge of PRV\textsubscript{TV} as early as 1 week post-immunization, highlighting the potential of rPRV\textsubscript{TV}-delgE as a vaccine for emergency immunization.

In summary, we developed a novel gE-deleted PRV, which is very safe and can provide complete protection against challenge with the emergent PRV variant from Bartha-K61-vaccinated swine population in China. Further studies are needed to evaluate the duration of immunity and efficacy for pregnant sows and newborn piglets and to elucidate the differences in protection between rPRV\textsubscript{TV}-delgE and Bartha-K61.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.04.035.

Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (TCID\textsubscript{50})</th>
<th>Days to fever onset</th>
<th>Fever frequency ≤40.5 °C</th>
<th>Fever frequency 40.5–41.0 °C</th>
<th>Fever frequency ≥41 °C</th>
<th>Survival rate</th>
<th>Viral shedding</th>
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<td>-</td>
<td>3</td>
<td>3/42</td>
<td>3/3</td>
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<tr>
<td>rPRV\textsubscript{TV}-delgE</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>0/75</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>rPRV\textsubscript{TV}-delgE</td>
<td>10\textsuperscript{4}</td>
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<td>0/75</td>
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<tr>
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<td>-</td>
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<td>-</td>
<td>15/42</td>
<td>1/3</td>
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</tr>
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<td>PBS</td>
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<td>4</td>
<td>7</td>
<td>4</td>
<td>15/42</td>
<td>1/3</td>
<td>3/3</td>
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</table>

Twenty-one 6-week-old healthy pigs from a PRV-free swine herd were randomly allocated into five groups. Pigs in Group A (n = 3) were inoculated intramuscularly (i.m.) with one-dose (10\textsuperscript{5} TCID\textsubscript{50}) of the PRV vaccine strain Bartha-K61. Groups B and C (n = 5) were immunized i.m. with 10\textsuperscript{5}, 10\textsuperscript{4} and 10\textsuperscript{3} TCID\textsubscript{50} of PRV\textsubscript{TV}-delgE, respectively. Group E (n = 3) were injected i.m. with PBS as control. One week post-immunization, all pigs were challenged with 10\textsuperscript{5} TCID\textsubscript{50} PRV\textsubscript{TV}. Following challenge, clinical signs and rectal temperature were recorded daily throughout the experiment. At 14 DPC, all pigs were euthanized and submitted to pathological and immunohistochemistry examinations.

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