RR1 and *RR2* gene deletion affects the immunogenicity of a live attenuated pseudorabies virus vaccine candidate in natural pig host

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Abstract As virulence-determining genes, *RR1* and *RR2* encode the small subunit and large subunit of viral ribonucleotide reductase (RR) in pseudorabies virus which have been extensively studied in mice. However, their role in pigs has not been adequately investigated. In this study, we deleted *RR1* and *RR2* genes based on a *TK*/*gE*/*gI* triple gene-deleted pseudorabies virus and tested its efficacy in pigs as a vaccine candidate. The rescued virus showed similar growth properties and plaque size *in vitro* as its parent strain. In an animal study, the virus could elicit humoral immune responses shown by generation of gB-specific antibodies and virus neutralizing antibodies. However, vaccination could not provide protection against virulent pseudorabies virus challenge since vaccinated pigs showed clinical pseudorabies-specific syndromes. The deficiency in protection may due to the generation of late and low levels of gB antibodies and virus neutralizing antibodies.

Keywords pseudorabies virus, *RR1* and *RR2*, ribonucleotide reductase, vaccine candidate

1   Introduction

Pseudorabies (PR) is an acute disease which has a wide range of hosts including ruminants, carnivores, rodents and pigs[1]. The causative agent, pseudorabies virus (PRV), belongs to family Herpesviridae, subfamily Alphaherpesvirinae, and is placed in the genus *Varicellovirus*. PRV has been successfully eradicated in western countries by using gene-deleted modified live vaccines accompanied by the application of ELISA tests[2]. However, with the emergence of PRV variants, this disease is still prevalent in China and poses a huge threat to the swine industry[3].

As for PRV modified live vaccines, several virulence-related genes such as thymidine kinase (TK), and glycoproteins glycoprotein E (gE) and glycoprotein I (gI) are commonly targeted for deletion to reduce virulence but keep immunogenicity[4]. By using bacterial artificial chromosome (BAC) technology, double- or triple-gene deleted vaccines were constructed and showed to provide useful protection as vaccine candidates[5,6]. In PRV, ribonucleotide reductase (RR) genes including *RR1* (UL39) and *RR2* (UL40) encode ribonucleotide reductase that reduces ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates and is a determinant of virus virulence in mice[7]. However, the roles of *RR* genes in PRV in the natural pig have seldom been reported. In this study, we successfully rescued a *RR1*/*RR2*-deleted virus based on previously constructed *TK*/*gE*/*gI* triple gene-deleted pseudorabies virus and tested its efficacy on pigs as a vaccine candidate.

2   Materials and methods

2.1   Construction of gene-deleted virus

The construction of *TK*/*gE*/*gI* triple gene-deleted pseudorabies virus (vPRV) has been described previously[4]. This strategy was used to construct *RR* gene-deleted virus based on the triple gene-deleted virus. Briefly, pBAC-PRV TK⁻/gE⁻/gI⁻ plasmid was transformed into *Escherichia coli* DY380 and positive colonies were made into

competent cells. A PCR product containing the kanamycin resistant gene with short sequence duplication of PRV *RR* genes was then transformed into the above competent cells. After digestion with I-*Sce*I, the linear plasmid was again transformed into *E. coli* DY380 to remove the kanamycin gene and to construct pBAC-PRV TK⁻/gE⁻/gI⁻/RR⁻ plasmid. To remove the BAC gene cassette, the plasmid was co-transfected with pBS 185 plasmid in Vero cells and *TK/gE/gI/RR* gene-deleted virus (vPRV TK⁻/gE⁻/gI⁻/RR⁻) was obtained after purification by plaque assay. Plaque sizes were determined at 48 h by inoculating 1000TCID$_{50}$ of virus on Vero cells. After 1 h incubation with virus, the medium was aspirated and cells were overlaid with 1% low-melting point agarose containing 2% FBS in DMEM for plaque formation. For each virus, 100 plaques were randomly selected and their size determined by ImageJ software (National Institutes of Health). Values were calculated in comparison to those of *TK/gE/gI* triple gene-deleted PRV which was set at 100%. Average percentages and standard deviations were determined from three independent experiments[4].

2.2 Animals and experiment design

Fifteen 3-week-old piglets free of PRV, porcine reproductive and respiratory syndrome virus, classical swine fever virus, and porcine circovirus 2 were randomly divided into 3 groups. In the first two groups, pigs were vaccinated intranasally with 10⁷ TCID$_{50}$ vPRV TK⁻/gE⁻/gI⁻ or vPRV TK⁻/gE⁻/gI⁻/RR⁻, respectively. Pigs in the unvaccinated group (CC group) received Dulbecco minimum essential medium. After vaccination, rectal temperature and clinical signs were recorded on a daily basis. The clinical score (range from 1 to 4) was evaluated on a daily basis as previously described[8]. Four weeks after vaccination, all pigs were challenged intranasally with 1 × 10⁷ TCID$_{50}$ virulent PRV variant HN1201. At 7 days post-challenge (dpc), all surviving pigs were euthanized and necropsied. The animal trial was approved by the Animal Care and Ethics Committee of China National Research Center for Veterinary Medicine.

2.3 Enzyme-linked immunosorbent assay and serum-virus neutralizing test (SNT)

The serum samples were collected to monitor PRV gB-specific antibody responses at designated days according to the manufacturer's directions (IDEXX, Westbrook, ME, USA). The cutoff S/N values for the assay are: negative as greater than 0.70, positive as less than or equal to 0.60, and suspect as greater than 0.60 and less than or equal to 0.70.

Serum samples were tested by SNT for the PRV-specific neutralizing antibodies. Briefly, serum samples were heat inactivated at 56°C for 30 min prior to performing the serum-neutralization assay. Two fold serially diluted sera (50 μL) were mixed with an equal volume 100 TCID$_{50}$ of

the PRV HN1201 in 96-well culture plates and incubated at 37°C for 1 h in 5% CO_2 atmosphere. After incubation, 100 μL of PK-15 cell suspension containing 2 × 10⁴ cells was added to each well. The inoculated cells were then incubated at 37°C for 5 days for development of CPE 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.

2.4 Histopathology and immunohistochemistry staining

The tonsil, lung, brain, cerebellum and trigeminal samples were collected for hematoxylin and eosin (H&E) and immunohistochemistry staining. The H&E staining was operated automatically by a Leica fully automatic dyeing machine according to standard procedures. The immuno-histochemistry staining was performed as previously described[9]. Photographs of the slides were taken at 400 × magnification.

2.5 Statistical analysis

Data in this study was presented as mean±SD. The differences of raw data among three groups were determined by using one-way ANOVA in GraphPad Prism 5.0 Software (San Diego, CA, USA). Differences were considered statistically significant when $P < 0.05$.

3 Results

3.1 Growth properties of gene-deleted virus

Virus growth was delineated by determination of virus titer every 4 h post-infection. The growth features of vPRV TK⁻/gE⁻/gI⁻/RR⁻ were virtually identical to parental vPRV TK⁻/gE⁻ gI⁻ in Vero cells as shown by Fig. 1a. The virus titers of these two viruses kept increasing and reached the maximum titers at 40 h post-infection. The plaque sizes of these two viruses were also determined at 48 h. As shown by Fig. 1b, the plaque sizes of reconstituted virus were similar to parental virus.

3.2 Antibody response after vaccination

After vaccination, no clinical symptoms were observed in any group of pigs (data not shown). gB-specific IDEXX ELISA showed that all pigs in the vPRV TK⁻/gE⁻/gI⁻ group were positive at 14 days post-caccination (dpv) and three pigs in the vPRV TK⁻/gE⁻/gI⁻/RR⁻ group were positive (Fig. 2a). At 28 dpv, all pigs in both vaccinated groups were positive for gB antibodies, and S/N value for the vPRV TK⁻/gE⁻/gI⁻/RR⁻ group was significantly higher than for the vPRV TK⁻/gE⁻/gI⁻ group (Fig. 2b). Unvaccinated pigs did not develop gB antibodies. Consistent with

gB ELSIA antibodies, the virus neutralizing antibody titers in the vPRV TK⁻/gE⁻/gI⁻/RR⁻ group were also significantly lower than vPRV TK⁻/gE⁻/gI⁻ group (Fig. 2c).

3.3 Clinical manifestations after viral challenge

After viral challenge, all pigs in the DMEM group showed typical PR-clinical symptoms such as fever (Fig. 3a), respiratory distress, excessive salvation and neurological signs. Also, all pigs in the vPRV TK⁻/gE⁻/gI⁻/RR⁻ group showed similar clinical manifestations as the pigs in the DMEM group with significantly higher clinical scores than for the vPRV TK⁻/gE⁻/gI⁻ group (Fig. 3b). In contrast, pigs in the vPRV TK⁻/gE⁻/gI⁻ group only showed a transient fever without other obvious clinical symptoms. No pigs were found to have died by 5 dpc in the vPRV TK⁻/gE⁻/gI⁻/RR⁻ group (Fig. 4a). However, three pigs had died at 3 dpc and two pigs died at 4 and 5 dpc in the DMEM group. All pigs were weighed at 4 dpc. As shown in Fig. 4b, all pigs in the DMEM and vPRV TK⁻/gE⁻/gI⁻/RR⁻ groups lost
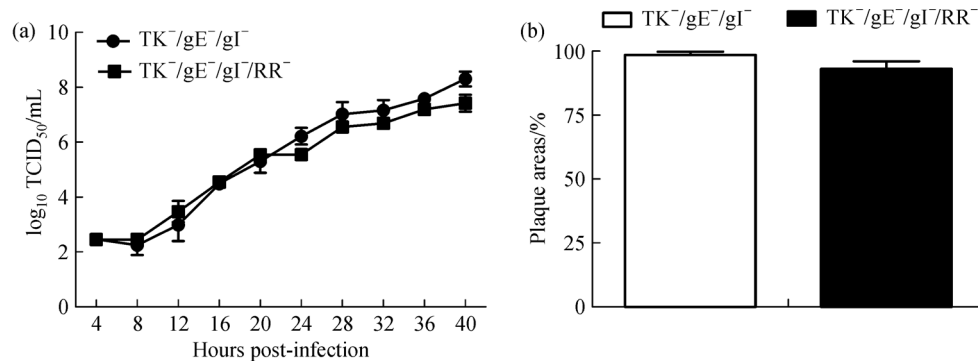

Fig. 1 Replication kinetics (a) and plaque size (b) of vPRVTK⁻/gE⁻/gI⁻/RR⁻. The virus titer at each time points and plaque size were averaged by triple samples.
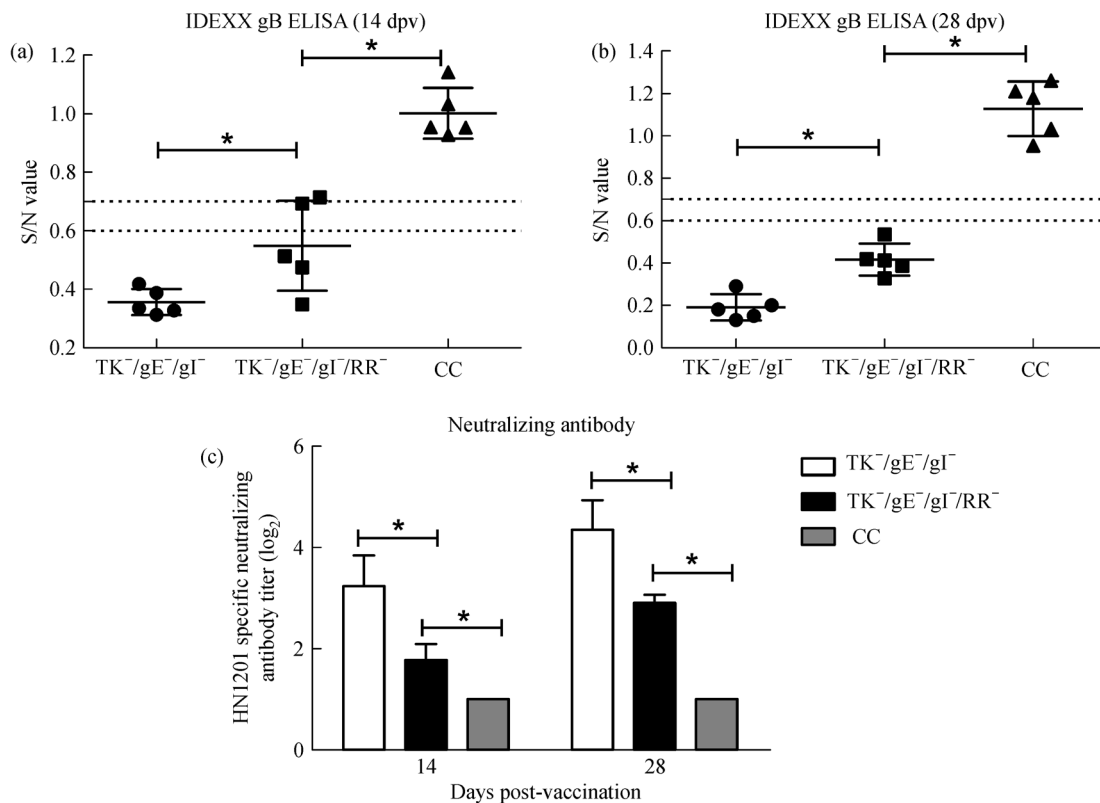

Fig. 2 PRV gB ELISA Ab responses (a, b) and PRV-specific virus neutralizing antibodies (c). The cutoff S/N values for the gB ELISA are: negative as greater than 0.7, positive as less than or equal to 0.6, and suspect as greater than 0.6 and less than or equal to 0.7. *, $P < 0.05$.
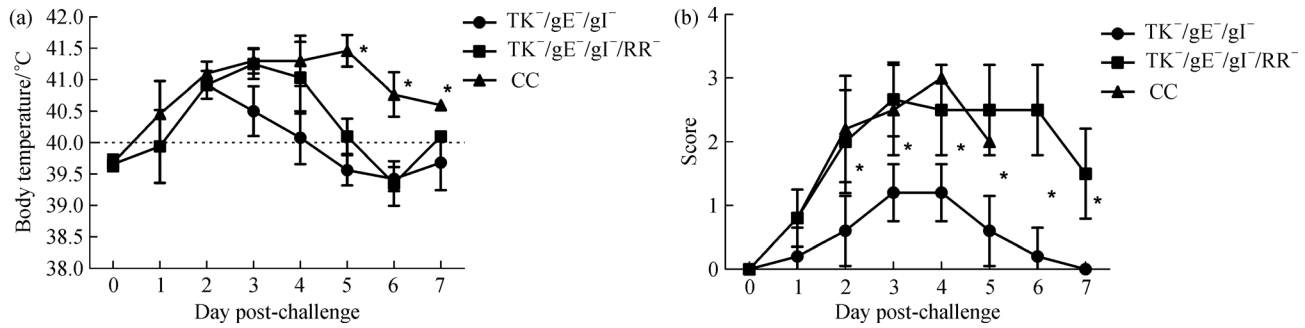
Fig. 3   Body temperature (a) and clinical symptom scores (b) of pigs after viral challenge. *, $P < 0.05$.

bodyweight, with daily bodyweight loss in the DMEM group significantly higher than that in the vPRV TK⁻/gE⁻/gI⁻/RR⁻ group. In contrast, pigs in the vPRV TK⁻/gE⁻/gI⁻ group had normal daily bodyweight gain.

3.4   Histopathology and immunohistochemistry staining

At necropsy, all pigs in the vPRV TK⁻/gE⁻/gI⁻/RR⁻ group and DMEM groups had hemorrhage and necrosis in tonsil samples, severe pulmonary consolidation and necrosis in lung samples, and encephalic hemorrhage in brain samples. Histopathological examination confirmed hemorrhages and necrosis in tonsil and lung samples in the vPRV TK⁻/gE⁻/gI⁻/RR⁻ and DMEM groups (Fig. 5). Neuronal degeneration and necrosis in brain and cerebellum were also observed in these two groups of pigs. Immunochemistry results showed positive staining in tonsil, lung, brain, and cerebellum samples (Fig. 5). There were no visible gross pathological and histopathological changes or positive staining of IHC for pigs in the vPRV TK⁻/gE⁻/gI⁻ group.

4   Discussion

Gene-deleted PRV live vaccines, combined with the use of an ELISA kit which differentiated vaccinated pigs from infected ones have been widely used to eradicate diseases. A good example is PRV Barth K-61 vaccine with *gE*/*gI*/*US9* gene-deletion that renders the strain incapable of anterograde spread[10]. Besides natural deletions of genes by extensively passaging field isolates in cell cultures, attenuation of PRV is also mediated by deletion of single or multiple virulence-determined genes such as *TK*, *gE* and *gI* by using BAC technology[4].

In PRV, ribonucleotide reductase catalyzes the reduction of ribonucleotides into deoxyribonucleotides, the substrates for DNA synthesis[8]. Previous studies showed that Herpes simplex virus (HSV) strains with deletion of *RR1* or *RR2* could replicate well in cell culture but were severely attenuated in mice[11]. One study also showed that *RR1*-deficient PRV was avirulent for pigs but induce partial

protective immunity[12]. Therefore, in this study, we completely removed *RR1* and *RR2* genes to evaluate deficient PRV efficacy as a vaccine candidate.

The rescued vPRV TK⁻/gE⁻/gI⁻/RR⁻ showed similar growth kinetics and plaque size as parental virus which indicates *RR* genes did not affect the proliferation of virus in dividing cells as previously reported[13]. However, vaccination did not provide protection against virulent PRV HN1201 challenge as all vaccinated pigs showed PR-typical clinical symptoms with high mortality. Compared to its parental virus, vPRV TK⁻/gE⁻/gI⁻/RR⁻ vaccinated pigs developed late and low level of gB-specific antibodies and virus neutralizing antibodies after vaccination. gB antibodies and virus neutralizing antibodies have been widely used to evaluate the efficacy of PRV vaccines, and inability to generate early and protective level of these antibodies may be due to the poor replication of virus via intranasal inoculation.

A *RR1*-deficient HSV-1 mutant showed poor replication in non-dividing cells[14]. However, the virus growth in dividing cells was found to be much better given that deoxyribonucleotides for virus replication were provided by cellular nucleotide metabolism[13]. In pigs, a previous study reported *RR1*-deleted PRV mutants had poor replication in nasal and oropharyngeal mucosa but did induce low titers of neutralizing antibodies which indicated vaccination could elicit immune responses[12]. Therefore, low proliferation of virus after vaccination may partially explain the generation of late and low level of gB-specific antibodies which could lead to failure of vaccination. However, we cannot exclude the possibility that some other factors may have contributed to the observed lack of protection.

In de Wind's study, a *RR1* mutant PRV provided protection against wild-type PRV challenge which differs from our results[12]. This discrepancy could be explained by different deletion of *RR* genes (both *RR1* and *RR2* genes were deleted in our study) and different parental viruses used for the gene manipulation (TK⁻/gE⁻/gI⁻ PRV was used in this study). However, the mechanisms of inability of this virus to provide protective immunity still need further exploration.
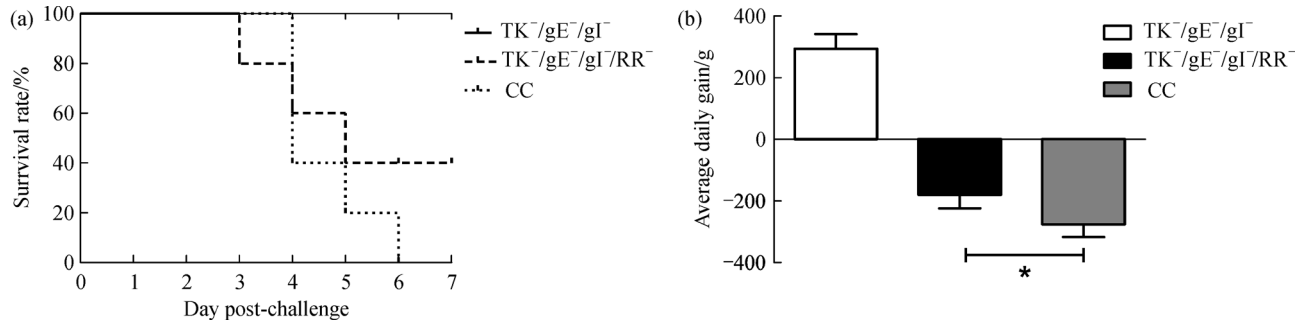
Fig. 4 Survival rate (a) and daily bodyweight gain for 4 dpc (b) of pigs after viral challenge. *, $P < 0.05$.
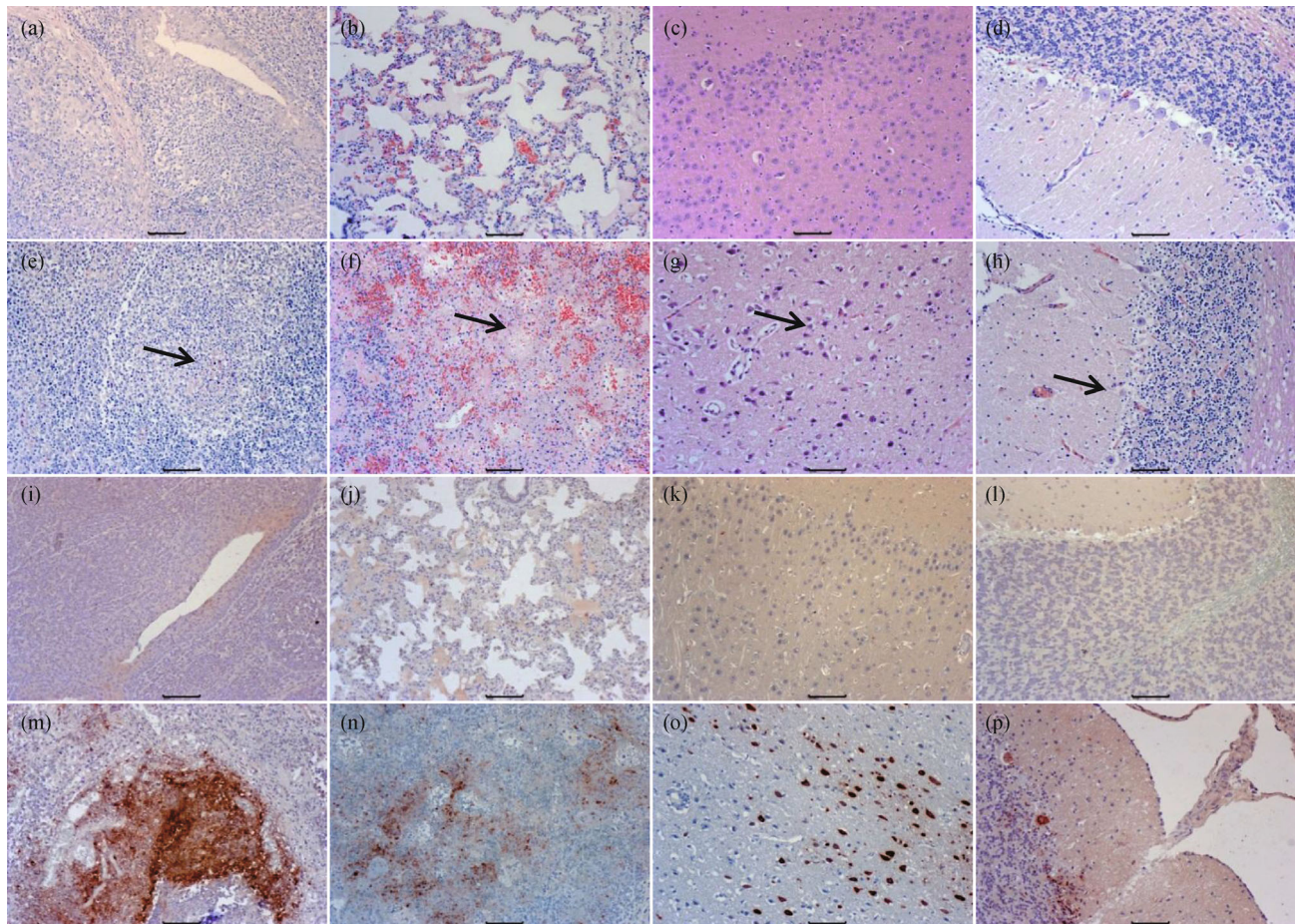

Fig. 5 Histopathological (a–h) and immunochemistry (i–p) results of vPRVTK⁻/gE⁻/gI⁻ (a–d, i–l) and vPRVTK⁻/gE⁻/gI⁻/RR⁻ (e–h, m–p). (a–d) Tonsil, lung, brain, and cerebellum samples from the pigs in vPRVTK⁻/gE⁻/gI⁻ group. No obvious histopathological changes were observed; (e) tonsil. Tonsillar lymphoid tissue necrosis and formation of a big necrotic foci; (f) lung. Massive vascular congestion and hemorrhage; (g) brain. Nonsuppurative meningoencephalitis; (h) cerebellum. Purkinje cell degeneration and necrosis; (i–l) negative immunochemistry staining of corresponding tonsil, lung, brain, and cerebellum samples in vPRVTK⁻/gE⁻/gI⁻ group; (m–p) positive immunochemistry staining of corresponding tonsil, lung, brain, and cerebellum samples in vPRVTK⁻/gE⁻/gI⁻/RR⁻ group. Original magnification 200×.

5   Conclusions

To conclude, we successfully constructed the *RR1* and *RR2* gene-deleted PRV strain based on vPRV TK⁻/gE⁻/gI⁻ virus and showed it was not effective as a vaccine candidate.

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