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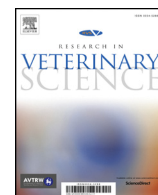

Comparative pathogenicity and immunogenicity of triple and double gene-deletion pseudorabies virus vaccine candidates

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A B S T R A C T

Pseudorabies (PR) outbreaks have re-emerged in many pig farms with vaccination coverage in China, which suggests that current vaccine strains provide poor protection against novel, epidemic strains. In this study, based on the highly virulent PRV ZJ01 variant, a PR virus (PRV) thymidine kinase (TK)/gE/gI deleted strain—rZJ01ΔTK/gE/gI—was generated, which showed similar growth features in vitro compared to the parent strain PRV ZJ01 and its gE/gI deleted strain rZJ01ΔgE/gI. The results of a piglet experiment (with 10 piglets each group) showed that the rZJ01ΔTK/gE/gI vaccine generated similar levels of neutralizing antibodies against ZJ01 compared to the rZJ01ΔgE/gI vaccine ($p > 0.05$). However, rZJ01ΔgE/gI inoculation resulted in slight inflammatory cell infiltrations, hemorrhages, and congestion in the brain and lungs. After a ZJ01 challenge, all animals in the rZJ01ΔTK/gE/gI- and rZJ01ΔgE/gI-vaccinated groups survived without exhibiting any clinical symptoms, whereas all non-vaccinated control animals died within 7 days post-challenge. Furthermore, microscopic lesions and virus loads in the brains and lungs in the two vaccinated groups were significantly lower than those in the control group. Meanwhile, the virus levels in the brains of piglets in the rZJ01ΔTK/gE/gI group were significantly lower than those in the rZJ01ΔgE/gI group. These results indicate that the triple gene-deleted PRV rZJ01ΔTK/gE/gI strain has lower pathogenicity and higher protective efficacy against variant PRV challenge compared with the double gene-deleted PRV rZJ01ΔgE/gI strain. Together, all these data indicate that the PRV rZJ01ΔTK/gE/gI strain should be an ideal vaccine candidate for the prevention of PR in China.

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1. Introduction

Pseudorabies virus (PRV), also named as *Suid alphaherpesvirus* 1, the causative agent of Pseudorabies (PR), is a member of the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus* (Pomeranz et al., 2005). Numerous symptoms are caused by this virus, including fatal encephalitis in newborn piglets, respiratory disorders in growing-fattening pigs, and reproductive failure in sows, resulting in heavy economic losses worldwide (Klupp et al., 2004).

Vaccination is one of the most efficient methods to control this disease. The gE-deleted PRV live vaccines have been broadly used to control this disease in China for a long time (Dong et al., 2014). However, since late 2011, massive PR outbreaks have been reported among PRV-vaccinated pig farms in China, which were probably caused by novel, highly pathogenic PRV variants with unique molecular signatures (An et al., 2013; Gu et al., 2015b; Luo et al., 2014; Wu et al., 2013; Ye et al., 2015; Yu et al., 2014). Therefore, it is necessary to develop more efficacious vaccines based on current epidemic PRV isolates. Recently, some live gE/gI-deleted and TK/gE/gI triple gene-deleted vaccines had been constructed from novel PRV variants. They could provide higher protection against the novel PRV variant challenge, comparing with the commercial Bartha-K61 vaccine (Wang et al., 2014; Zhang et al., 2015). But the pathogenicity and immunogenicity of double and triple gene-deletion virus vaccine candidates have not been completely understood.

In our previous studies, we isolated and characterized a highly virulent PRV variant ZJ01, and sequence analysis indicated that it is closely related to other recently emergent PRV variants that have been isolated by different research groups in China (Gu et al., 2015b). And a novel inactivated gE/gI-deleted PRV vaccine rZJ01ΔgE/gI was constructed based on the PRV ZJ01 variant (Gu et al., 2015a). In this study, a thymidine kinase (TK) and gE/gI-deleted PRV construct, rZJ01ΔTK/gE/gI, was generated from the gE/gI-deleted PRV strain rZJ01ΔgE/gI, and its pathogenicity, immunogenicity, efficacy and clinical protection were comparatively evaluated in piglets. The results showed that the triple gene-

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deleted variant strain could provide full protection against challenge with the highly virulent PRV ZJ01 strain, and that it is a better vaccine candidate than the double gene-deleted mutant.

2. Materials and methods

2.1. Viruses and cells

The highly virulent PRV variant ZJ01 (5th culture passage) was isolated in 2012 from an affected pig farm in Zhejiang, China (Gu et al., 2015b). rZJ01ΔgE/gI (6th culture passage), in which the gI and gE genes are deleted, was constructed in our laboratory, as described previously (Gu et al., 2015a). The PRV LA strain used in neutralizing antibody (NAb) tests, is a traditional Chinese strain isolated in 1997. Virus propagations, titrations, growth kinetics measurements, and plaque purifications were performed in baby hamster kidney-21 (BHK-21) cells. BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 1% penicillin and streptomycin (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C in 5% CO_2 in a humidified incubator.

2.2. Primers and plasmids

The primer pairs PRV-H1F/H1R and H2F/H2R (Table S1) were designed to amplify the left and right homologous arms, respectively, of the ZJ01 genome flanking the UL23 gene that encodes TK. Both fragments are approximately 1.1 kb in length. The fragments were cloned into the pUC19 vector (Invitrogen, Carlsbad, CA, USA) using restriction enzyme sites in the primers creating pUC19-H1-H2. A mini-F vector containing *Escherichia coli* xanthine guanine phosphoribosyl transferase (Eco-gpt) and enhanced green fluorescent protein (eGFP) gene was released as a *Pac*I fragment from plasmid pDS-pHA2 and cloned into the *Pac*I site in pUC19-H1-H2 to construct the transfer plasmid pHA2-pUC19-H1-H2 (Fig. 1D) (Schumacher et al., 2000; Wang and Osterrieder, 2011).

2.3. DNA extraction and transfection

The rZJ01ΔgE/gI virus was propagated in BHK-21 cells and purified using 20–60% sucrose density gradient centrifugation (130,000 × g for 2 h at 4 °C). Viral DNA was purified using previously described sodium dodecyl sulfate (SDS)–proteinase K extraction method (Smith and Enquist, 1999). Transfection of plasmid or virus DNA was achieved using Lipofectamine® 3000 reagent (Invitrogen) according to the manufacturer's instructions.

2.4. Generation of virus mutants

Virus mutants were generated using a previously described homologous recombination method, with some modifications (Schumacher et al., 2000; Wang and Osterrieder, 2011). Briefly, rZJ01ΔgE/gI genomic DNA (gDNA) and pHA2-pUC19-H1-H2 were co-transfected into BHK-21 cells (5×10^5 cells/well). When a cytopathic effect (CPE) was observed, the transfected culture was overlaid with DMEM medium containing 2% fetal bovine serum and 1% agarose. When green fluorescent plaques were recognized, plaque purification was conducted to obtain the homogeneous viruses. After multiple rounds of plaque purification, a reconstituted virus that lacked the TK gene and expressed green fluorescence was obtained. The presence of the gB gene and the absence of the TK gene were verified by polymerase chain reaction (PCR) using gB-specific (gBdF/gBdR) and TK-specific (TKdF/TKdR) primer pairs (Table S1), respectively, and nucleotide sequencing reactions were performed by Invitrogen (Shanghai, China). The expected TK-deleted virus expressing eGFP, which was derived from the rZJ01ΔgE/gI strain, was named ZJ01ΔTK/gE/gI-eGFP. The TK/gE/gI- deleted, eGFP-free PRV mutant rZJ01ΔTK/gE/gI was generated with co-transfection of ZJ01ΔTK/gE/gI-eGFP gDNA and pUC19-H1-H2 into BHK-21 cells, as described above (Fig. 1). Non-fluorescent plaques were purified to obtain a homogeneous virus population.

2.5. PRV one-step growth kinetics and plaque size determination

One-step growth kinetics and plaque sizes of all the viruses used in the experiment were measured as previously described (Gu et al., 2015a).

2.6. Immunofluorescence assay (IFA)

To check the expression of the gB protein, BHK-21 cells were infected with the rZJ01ΔTK/gE/gI, rZJ01ΔgE/gI, or ZJ01 strains at an MOI of 1 for 18 h. Cells were fixed with cold ethanol for 30 min. The fixed cells were incubated with a mouse anti-gB monoclonal antibody (mAb) (1:10,000 dilution, previously made by our lab) for 1 h at 37 °C in a humidified chamber, followed by three washes with phosphate-buffered saline (PBS). Then, the cells were incubated with fluorescein

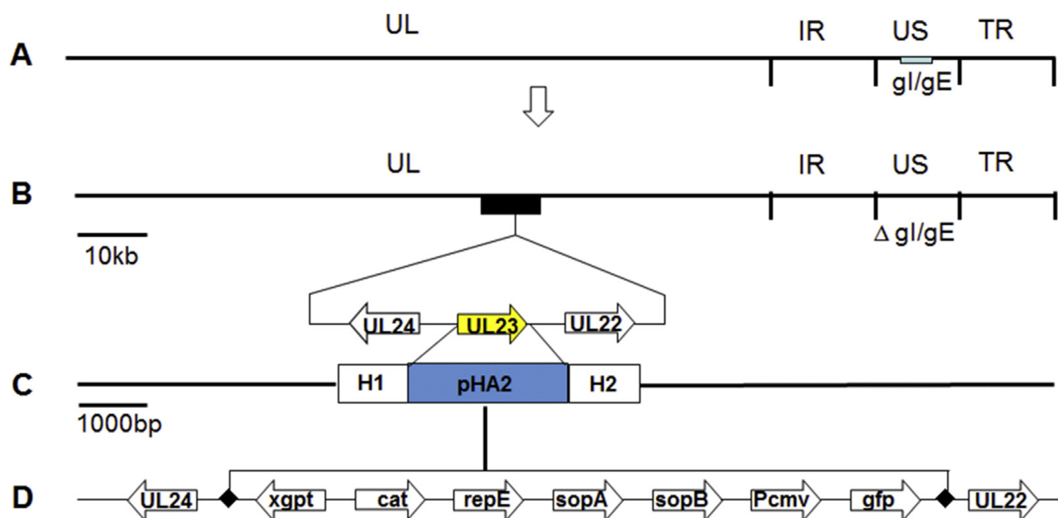

Fig. 1. Strategy for the construction of the rZJ01ΔTK/gE/gI-eGFP strain. (A) Organization of the ~140 kb ZJ01 genome. (B) Organization of the ~140 kb rZJ01ΔgE/gI genome (Gu et al., 2015a, b). (C) Sites of gene replacements and the genome organization of the rZJ01ΔTK/gE/gI-eGFP strain. (D) Elements of plasmid pHA2. UL, unique long region; IR, internal repeat; US, unique short region; TR, terminal repeat.

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