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Pathogenicity and immunogenicity of a gE/gI/TK gene-deleted pseudorabies virus variant in susceptible animals

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ABSTRACT

A pseudorabies virus (PRV) variant with enhanced pathogenicity has emerged in many vaccinated swine herds in China since 2011. rPRVTJ-delgE/gI, a previously described gE/gI-deleted PRV based on the PRV variant TJ strain, has been shown to be avirulent to pigs yet virulent to sheep. To ensure desirable biosafety, we further deleted the thymidine kinase (TK) gene of rPRVTJ-delgE/gI to generate a gE/gI/TK-deleted mutant rPRVTJ-delgE/gI/TK, and evaluated its pathogenicity and immunogenicity in susceptible animals. Groups of mice ($n=5$), sheep ($n=3$), and pigs ($n=4$) were inoculated with different doses of rPRVTJ-delgE/gI/TK or rPRVTJ-delgE/gI, and clinical signs, viral shedding, pathological changes, and serum antibodies were examined following inoculation. The results showed that rPRVTJ-delgE/gI/TK displayed higher safety than rPRVTJ-delgE/gI for mice (10^3–10^6 $TCID_{50}$) and sheep (10^5 $TCID_{50}$), and pigs inoculated with rPRVTJ-delgE/gI/TK (10^5 $TCID_{50}$) induced PRV-specific antibodies and protection against lethal PRV challenge comparable to those immunized with rPRVTJ-delgE/gI. In conclusion, rPRVTJ-delgE/gI/TK has the potential to be developed as a vaccine for controlling the currently prevalent PR in China.

1. Introduction

Pseudorabies (PR), which is caused by pseudorabies virus (PRV), is an economically important viral disease in pigs and other animals (McGregor et al., 1985). PRV is a member of the *Alphaherpesvirinae* subfamily in the *Herpesviridae* family, and has a broad host range in domestic and wild animals, with the pig being its primary natural host and reservoir. Like other alpha-herpesviruses, PRV usually establishes a life-long latent infection in the host's peripheral nervous system. Latently infected pigs can be a source of reinfections when the latent viral genome is reactivated spontaneously.

PR has been eradicated from domestic pig populations in many countries, including the United States and many European countries (Brittle et al., 2004; Klupp et al., 2004). Since mid-1980s, Bartha-K61-based vaccines have been widely applied in China, resulting in relatively favorable control of PR (Tong and Chen, 1999; Kong, 2000). Since 2011, however, increasing PR outbreaks have been reported in many pig farms in China, where regular vaccination has been carried out. Several studies have indicated that Bartha-K61 strain vaccine cannot provide complete protection against the emerging PRV variants, which exhibit high pathogenicity and unique molecular signatures (An et al., 2013; Tong et al., 2013; Yu et al., 2014; Luo et al., 2014). To overcome the current problems, several vaccines based on current PRV variants have been developed, including the gE/gI-deleted PRV based on the TJ strain (Wang et al., 2014), the gE/gI/TK-deleted PRV based on the PR HN1201 strain (Zhang et al., 2015), and the killed gE/gI-deleted PRV based on the ZJ01 strain (Gu et al., 2015). These vaccines were reported to provide effective protection aganist the PRV variants. However, data are not available about the safety of the above vaccines in other susceptible animals such as mice or sheep (an ideal model for PR vaccine evaluation). Recently, we demonstrate that the gE/gI-deleted PRV rPRVTJ-delgE/gI based on the TJ strain is able to induce disease and death in sheep (data not shown), which indicates that rPRVTJ-delgE/gI will be not applicable in some animal species. This phenomenon has been described by Kong et al. (2013), who demonstrated that an outbreak of PR in sheep was caused by a live attenuated vaccine, Bartha-K61. This indicates that the deletion of the gE and gI genes is not sufficient to attenuate the lethal PRV. Therefore, it is urgent to develop a safer yet effective vaccine for controlling the current PR in China.

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Besides the gE and gI proteins, thymidine kinase (TK) is also virulence-related and nonessential for the *in vitro* replication and the immunogenicity of PRV (Jacobs et al., 1993; Morenkov et al., 1997). As one of the viral enzymes, TK is involved in the viral replication and spread of PRV within the central nervous system (CNS) (Ferrari et al., 1998). It has been reported that partial or complete deletion in the TK gene may reduce virulence and impair neuroinvasiveness (Ferrari et al., 2000).

To avoid possible biosafety issues, here we generated a gE/gI/TK-deleted PRV mutant based on rPRVTJ-delgE/gI and evaluated its pathogenicity and immunogenicity in several susceptible animals including mice, sheep, and pigs.

2. Materials and methods

2.1. Viruses and cells

The PRV mutant rPRVTJ-delgE/gI derived from the TJ strain was described previously (Fig. 1) (Wang et al., 2014). The PRV TJ strain, a virulent PRV variant (Luo et al., 2014), was used for virulent challenge. These viruses were propagated and titrated in PK-15 or Vero cells, which were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine sera (FBS) (Gibco, USA), 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37 °C and 5% CO_2.

2.2. Construction of recombinant transfer vectors

For deletion of the TK gene of rPRVTJ-delgE/gI by homologous recombination, a transfer vector was constructed by using two segments flanking with the TK gene, TK-L and TK-R, as the recombination homologous arms. TK-L and TK-R were amplified from the PRV TJ genomic DNA using primer pairs P1S/P1R and P2S/P2R (Table 1). The fragments of TK-L and TK-R were cloned into the pOK12 vector (Novagene, USA) creating pOK-LR. The EGFP expression cassette under the control of the CMV promoter and SV40 poly(A) signal was amplified from pEGFP-C1 (Clontech, USA) with primers P3S and P3R (Table 1) and cloned into the *Mlu*I site of pOK-LR to construct the transfer vector pOK-LR-EGFP.

2.3. Generation of recombinant viruses

The genomic DNA of PRV TJ mutant rPRVTJ-delgE/gI was extracted and purified using the DNA Tissue Kit (Omega, USA) according to the manufacturer's instructions. The resulting genomic DNA (2 μg) and the transfer plasmid pOK-LR-EGFP (5 μg) were cotransfected into Vero cells (2×10^5 cells/well) using the X-tremeGENE HP DNA transfection reagent (Roche, USA) according to the manufacturer's instructions. After cytopathic effects (CPEs) were observed, the transfected cells were harvested, followed by two freeze-thaw cycles, inoculated in naïve PK-15 cells, and overlaid with 1% agarose-containing and 2% FBS in DMEM. Plaques with green fluorescence were purified by multiple rounds of screening in PK-15 cells. The expected gE/gI/TK-deleted virus expressing EGFP was generated and named as rPRVTJ-delgE/gI/TK-EGFP (Fig. 1). Similarly, rPRVTJ-delgE/gI/TK (Fig. 1) without EGFP gene was generated in Vero cells cotransfected with the plasmid pOK-LR and the genomic DNA of rPRVTJ-delgE/gI/TK-EGFP, as described previously. The absence of the PRV TK and gE/gI genes was verified by PCR and sequencing using P4S/P4R and P5S/P5R primers (Table 1).

2.4. One-step growth curves

The growth kinetics of rPRVTJ-delgE/gI/TK, rPRVTJ-delgE/gI, and PRV TJ strains were determined as described previously (Luo et al., 2014).

2.5. Experimental infections of mice

All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals of Harbin Veterinary Research Institute, CAAS, China.

Forty-five 6-week-old specific-pathogen-free (SPF) BALB/c mice were randomly divided into 9 groups of five mice each. Groups 1–8 were each inoculated intraperitoneally (i.p.) with 100 μl of different doses (10^3, 10^4, 10^5, or 10^6 $TCID_{50}$) of rPRVTJ-delgE/gI/TK or rPRVTJ-delgE/gI, and mice in group 9 were injected with DMEM serving as an uninfected control. Following inoculation, clinical signs were monitored daily, and the median lethal dose (LD_{50}) was calculated. At 14 days post-inoculation (DPI), all surviving mice were euthanized and the brain samples were collected and tested for the presence of PRV using the gB-specific PCR.

2.6. Inoculation of sheep with rPRVTJ-delgE/gI/TK

Fifteen healthy 18-month-old sheep were obtained from a local farm without PR history. The sheep were tested free of PRV by serum-virus neutralization test (SVNT), blocking enzyme-linked immunosorbent assay (ELISA) and PCR. All the sheep were

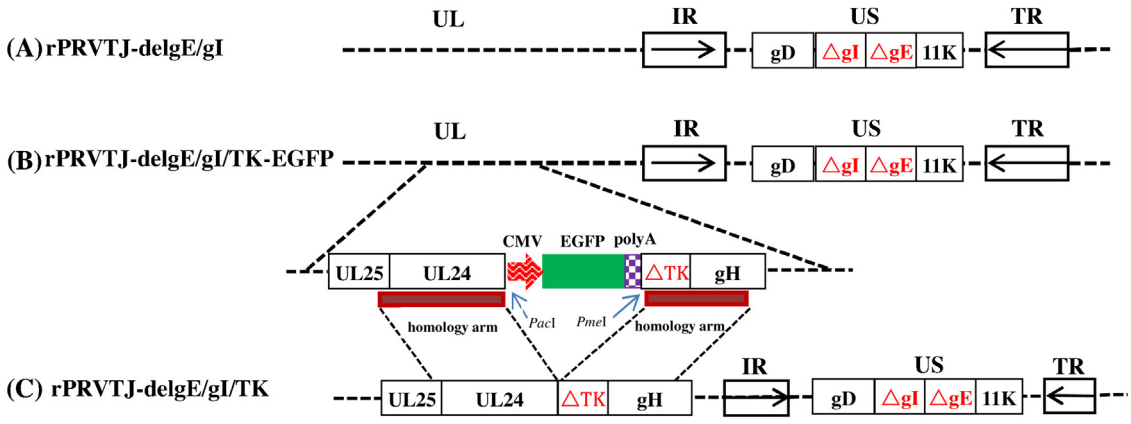

Fig. 1. Schematic diagrams of the PRV recombinants. (A) The schematic representation of rPRVTJ-delgE/gI genome in which partial coding regions of glycoprotein I (gI) and glycoprotein E (gE) genes are deleted. (B) The schematic representation of rPRVTJ-delgE/gI/TK-EGFP genome in which partial coding regions of gI, gE and thymidine kinase (TK) genes are deleted and an EGFP expression cassette is inserted into the TK deleted locus. (C) The schematic representation of rPRVTJ-delgE/gI/TK genome. CMV: human cytomegalovirus; polyA: SV40 poly(A) signal.