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A novel inactivated gE/gI deleted pseudorabies virus (PRV) vaccine completely protects pigs from an emerged variant PRV challenge

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ABSTRACT

A highly virulent and antigenic variant of pseudorabies virus (PRV) broke out in China at the end of 2011 and caused great economic loss in the pig industry. In this study, an infectious bacterial artificial chromosome (BAC) clone containing the full-length genome of the emerged variant PRV ZJ01 strain was generated. The BAC-derived viruses, vZJ01-GFPΔgE/gI (gE/gI deleted strain, and exhibiting green autofluorescence), vZJ01ΔgE/gI (gE/gI deleted strain), and vZJ01gE/gI-R (gE/gI revertant strain), showed similar *in vitro* growth to their parent strain. In pigs, inactivated vZJ01ΔgE/gI vaccine generated significantly high levels of neutralizing antibodies against ZJ01 compared with Bartha-K61 live vaccine ($p < 0.05$). After fatal ZJ01 challenge, all five animals in the inactivated vZJ01ΔgE/gI vaccine group survived without exhibiting any clinical sings, but two of five animals exhibited central nervous signs in the Bartha-K61 group. Meanwhile, all the non-vaccinated control animals died at 7 days post-challenge. This indicates that the inactivated vZJ01ΔgE/gI vaccine is a promising vaccine candidate for controlling the variant strains of PRV now circulating in China.

1. Introduction

Pseudorabies virus (PRV) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus*. It can cause fatal encephalitis in newborn pigs, respiratory disorders in growing–fattening pigs, and reproductive failure in sows, and causes great economic loss worldwide (Brittle et al., 2004; Klupp et al., 2003; Szpara et al., 2011). By using gE deleted PRV vaccines, the United States and some European countries have eradicated pseudorabies from their domestic pig populations (Brittle et al., 2004; Klupp et al., 2003). In China, PRV live vaccination has been widely used to control effectively this disease and the huge economic losses have been decreased during the past several decades.

However, since late 2011, a severe PRV outbreak took place in many pig herds immunized with PRV vaccines and spread widely to most pig farms in China (An et al., 2013; Wang et al., 2014; Wu et al., 2013; Yu et al., 2014). Novel antigenic variant PRV isolates have been isolated from the pigs with clinical signs (An et al., 2013; Wang et al., 2014; Wu et al., 2013; Yu et al., 2014). We also isolated a highly virulent PRV strain ZJ01 with antigenic variant from a pig herd in China in 2012. It could cause 100% mortality both in 14 and 80 days old pigs' experiments. The serum antibodies to the commercial PRV vaccines had significantly lower neutralizing activity to ZJ01 isolate, comparing with those to the original vaccine strains (Gu et al., 2014). The newly isolates had many specific amino acid mutations in gE and gC genes, compared with those in classical PRV strains of China and US and European strains (An et al., 2013; Gu et al., 2014; Yu et al., 2014). Thus, it is necessary to develop a new method to control this disease.

The application of bacterial artificial chromosome (BAC) technology to the study of herpes viruses has facilitated a variety of studies of proteomics, vaccines, and gene function using both forward and reverse genetics (Adler et al., 2001; Cicin-Sain et al., 2003; McGregor and Schleiss, 2004; Meseda et al., 2004; Schumacher et al., 2000; Wang and Osterrieder, 2011). In this study, the full-length genome of PRV was extracted and an infectious bacterial artificial chromosome (BAC) clone was constructed from PRV strain ZJ01 by utilizing efficient homologous recombination protocols. And a gE/gI deleted PRV strain vZJ01ΔgE/gI was generated from the PRV ZJ01 BAC. In order to control this emergence disease

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in China, we inactivated the recombinant PRV vZJ01ΔgE/gI with formalin and prepared killed vaccine with the mineral adjuvant MONTANIDE™ ISA 206. And it was found that the inactivated gE/gI deleted PRV vaccine could provide greater protection than Bartha-K61-vaccine against lethal ZJ01 challenge.

2. Materials and methods

2.1. Virus and cells

PRV strain ZJ01 (5th culture passage) was isolated in 2012 from an affected pig farm in Zhejiang Province, China, and propagated in BHK-21 cells. No difference in DNA sequence was observed in PCR products amplified from the 1st culture passage after plaque-purified and the 5th passage virus. BHK-21 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA, USA) supplemented with 8% fetal calf serum (Gibco) and 1% penicillin and streptomycin (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C with 5% CO_2 in a humidified incubator.

2.2. Primers and plasmids

PRV-HOMO1-for/PRV-HOMO1-rev and PRV-HOMO2-for/PRV-HOMO2-rev (Table 1) were designed to amplify the areas of PRV genome flanking the Us7 and Us8 genes encoding gI and gE, respectively. Both fragments were ~1.1 kbp in length. The fragments were cloned into pUC19 (Invitrogen, Carlsbad, CA, USA) in turn using restriction enzyme sites show in the primers and the resulting recombinant plasmid was named pUC19-H1-H2 (Fig. 1). A mini-F vector containing Eco-gpt and the GFP gene was released as a *Pac*I fragment from plasmid pDS-pHA2 (Schumacher et al., 2000) and cloned into the *Pac*I site present in pUC19-H1-H2 to construct transfer vector plasmid pHA2-pUC19-H1-H2.

2.3. DNA extraction and transfection

Preparation of plasmid or BAC DNA was done with commercially available kits (Qiagen, Valencia, CA). The ZJ01 strain was propagated in BHK-21 cells and then purified by using 20–60% sucrose density gradient centrifugation. Viral DNA was purified from infected cells or purified virus by SDS–proteinase K extraction as described earlier (Smith and Enquist, 1999). Transfection of plasmid, virus or BAC DNA was achieved using Lipofectamine 2000 reagent (Invitrogen), following the instructions of the manufacturer.

2.4. Bacterial manipulations

Chemically *Escherichia coli* cells Top 10 (Invitrogen) and electrocompetent MegaX DH10B $T1^R$ Electrocomp cells (Invitrogen) were used. Electroporation was carried out in 1-mm cuvettes at 1500 V/cm, resistance of 200 Ω, and capacitance of 25 μΩ (Genepulser Xcells; BioRad, CA, USA) (Schumacher et al., 2000; Wang and Osterrieder, 2011). Transformed bacteria were incubated in 1 mL SOC medium for 1 h at 37 °C and then plated on LB agar containing chloramphenicol and incubated at 37 °C.

2.5. Generation of a PRV BAC clone and mutant viruses

To generate the PRV BAC clone, a mini-F including PRV mutant was constructed following the general technique of BAC, as described previously (Schumacher et al., 2000; Wang and Osterrieder, 2011), with some modifications. BHK-21 cells were transfected with ~1 μg PRV ZJ01 viral DNA and 1–5 μg pHA2-pUC19-H1-H2 (Fig. 1). When a cytopathic effect (CPE) was observed, the transfected culture was harvested by trypsinization digestion, plated on the fresh BHK-21 cells, and overlaid with medium containing 1% agarose. When green fluorescent plaques were recognized, plaque purification was carried out to obtain the homogeneous viruses. Mini-F containing PRV mutant viruses were propagated on fresh BHK-21 cells. After 2 days post-infection, circular viral DNA was isolated and then transferred into MegaX DH10B $T1^R$ Electrocomp cells by electroporation. BAC DNA was isolated and separated by pulsed-field gel electrophoresis (PFGE) using CHEF Mapper XA Chiller System (Bio-Rad). PFGE patterns were detected by UV transillumination after SYBR-Gold (Invitrogen) staining.

To obtain PRV gE/gI-negative virus (vZJ01ΔgE/gI) without mini-F vector sequence, homologous recombination was used to replace the mini-F vector in the PRV genome with the pUC19-H1-H2 sequences. Nonfluorescent plaques were purified to get a homogeneous virus population.

To obtain the revertant virus (vZJ01gE/gI-R), homologous recombination was used in lieu of the mini-F vector in the PRV genome with the deleted gE and gI sequence, which was amplified from ZJ01 DNA by PCR with primers PRV-HOMO1-for/PRV-HOMO2-rev. Nonfluorescent plaques were purified to get a homogeneous virus population (vZJ01gE/gI-R).

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

One-step growth kinetics was conducted as described previously (Smith and Enquist, 1999). The culture supernatants were tested at 0, 4, 8, 12, 16, 20, 24, 28 and 32 h post-infection (hpi) by inoculating the cell monolayers at an MOI of 1. Virus titers presented as 50% tissue culture infectious dose ($TCID_{50}$). Growth kinetics for each virus tested was performed in duplicate, and the resulting titers were averaged.

Plaque sizes were determined at 48 h after plating 500 $TCID_{50}$ of the respective viruses onto BHK-21 cells seeded in six-well plates and after adding medium containing 1% agarose 1 h after plating.

Table 1
Oligonucleotide primers used in this study.

Primers	Primer sequences (5′–3′)[a]	Applications
PRV-HOMO1-for	*ATT***gaattc**GTACCCGTACACCGAGTCGT	To amplify H1 fragment
PRV-HOMO1-rev	*ACT***gagctc***GG***ttaattaa**TCATCATCGACGCCGGTACT	
PRV-HOMO2-for	*CAA***ctgcag***CG***ttaattaa**GCCGACATGGACACGTTCGA	To amplify H2 fragment
PRV-HOMO2-rev	*TCG***aagctt**TTGTGGACCCGCGCGAACAT	
PRV-gE-632-for	TCCACTCGCAGCTCTTCT	To detect gE gene
PRV-gE-632-rev	GCACGTCATCACGAAGGA	
PRV-gB-for	GTGTACTACGAGGACTACAGCTACGTGCGC	To detect gB gene
PRV-gB-rev	CAGGGCGTCGGGGTC	
PRV gE 694F	CTTCCACTCGCAGCTCTTCTC	Real-time PCR
PRV gE 765R	GTRAAGTTCTCGCGCGAGT	
Probe	FAM-TTCGACCTGATGCCGC-TARAM	

[a] Sequences in italics indicate additional bases which are not present in the PRV sequence and restriction sites are in bold lower case letters.