Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.053

RESEARCH ARTICLE

Construction of a Triple Gene-Deleted Live Vaccine Candidate against Pseudorabies Virus Using CRISPR/Cas9 and Cre/LoxP System-Based Strategy

Muping Wang, Longtao Wang* and Quan Zhao*

College of Animal Sciences and Technology, Jilin Agricultural University, 2888 Xincheng Street, Changchun 130118, China
*Corresponding author: zhaoquan0825@163.com; wanglongtao2005@126.com

ARTICLE HISTORY (21-137)

Received: April 03, 2021
Revised: June 09, 2021
Accepted: June 25, 2021
Published online: July 24, 2021

Key words:
Cre/Lox system
CRISPR/Cas9
PRV
Pseudorabies virus
Triple gene-deleted live vaccine

ABSTRACT

The immune protective effects of pseudorabies virus (PRV) vaccines in the market have gradually reduced and they have failed to provide complete protection against the new PRV variant. In this study, a triple gene-deleted live PRV strain–rZDΔTK-gE-gI was successfully constructed by simultaneously knocking out the three major virulence genes (gE/gI and TK) with CRISPR/Cas9 and Cre/LoxP gene editing system and low melting point agarose purification method. After challenge with the virulent PRV variant, all the 3-week-old piglets vaccinated with the rZDΔTK-gE-gI PRV vaccine candidate survived without any clinical symptoms, whereas all the unvaccinated piglets exhibited PRV respiratory and neurological signs with 100% mortality rate within 7 days post infection. High levels of anti-gB antibodies were induced in the vaccinated piglets after vaccination with the rZDΔTK-gE-gI vaccine candidate, which elicited a better immune protective effect than the classical strain Bartha-K61. Therefore, the triple gene-deleted live PRV vaccine candidate is expected to control the current outbreak of pseudorabies caused by the PRV variants.

To Cite This Article: Wang M, Wang L and Zhao Q, 2021. Construction of a triple gene-deleted live vaccine candidate against pseudorabies virus using CRISPR/Cas9 and Cre/LoxP system-based strategy. Pak Vet J, 41(4): 493-498. http://dx.doi.org/10.29261/pakvetj/2021.053

INTRODUCTION

Pseudorabies (PR) is an acute infectious disease caused by pseudorabies virus (PRV) in many domestic and wild animals (Fan *et al.*, 2016; Papageorgiou *et al*., 2016; Yu *et al.*, 2016; Masot *et al.*, 2017; Sun *et al.*, 2018; Wu *et al*., 2018). PRV belongs to the Family *Herpesviridae*, subfamily *Alphaherpesvirus* and genus *Varicellovirus*. The main clinical symptoms are fever, itching, encephalomyelitis, respiratory and nervous system disorders. Inactivated or attenuated live vaccines can be used to control pseudorabies in pigs. Classical attenuated PRV vaccines developed from field virulent PRV strains by deleting one or more genes have been a new tool for disease control. Firstly, the gene engineered live virus (labeled) vaccine with missing virulence determining gene is constructed, and then wild virus infection and labeled vaccine vaccination can be differentiated by the serological identification using the corresponding serological diagnostic methods. The foundation of this process is the molecular characteristics of PRV and the identification of nonessential envelope glycoproteins, such as glycoprotein gE, which can be deleted or removed from the virus without affecting the replication or immunogenicity of the virus to achieve the purpose of differential diagnosis (Ren *et al*., 2018) . The practice of vaccination to eradicate PR in pig farms has been successful in several countries, including Germany and the United States. Over the years, the new epidemic situation of pseudorabies has occurred and prevailed in many areas of China; it can be more severe in some provinces, with a large number of infected pig farms and huge loss. The antigenicity of the new epidemic strain has changed, and the pathogenicity of the new virus strain has increased. The existing attenuated vaccine like Bartha-K61 cannot fully prevent pigs from the infection of the epidemic variants (Hu *et al.*, 2015). The PRV variants were found to be more virulent than the classical PRVs in piglets of different ages (Tong *et al.*, 2015; Yang *et al.*, 2016). Therefore, it is necessary to develop a new vaccine against PRV variant strain.

With the continuous progress and advance of molecular biology and gene engineering technology, the molecular biological characteristics and gene functions of PRV have been completely analyzed. Lack of one or more non-essential virus replication genes can greatly reduce

the virulence of PRV, but it does not affect its proliferation and immunogenicity (Wang *et al.*, 2015; Cong *et al.*, 2016) . CRISPR/CAS system, an innovative and powerful genome editing tool, has been widely used in many research fields. The endonuclease Cas9 protein identifies specific genomic sites through guide RNA (gRNA) and cleaves double stranded DNA (Ma *et al.*, 2014) . Cells then repair the cleaved sites by non-homologous end joining (NHEJ) or homologous recombination (HR) to achieve DNA level gene knockout or precise editing. CRISPR/CAS system uses the interaction between RNA and DNA to locate gene in the genome. Only a small RNA sequence is needed to anchor the target gene position, which greatly reduces the workload of gene editing and has high specificity. In addition, Cre LoxP system is a recombinant enzyme system, which can control the occurrence of site-specific recombination in the genomic DNA (Van Duyne, 2015) . It is widely used in gene knockout, insertion, turnover and translocation of specific sites, and can achieve the goal of targeted genetic transformation of organisms at the gene level. It is mainly composed of Cre and LoxP. Cre recombinase can effectively knock out the sequence between two LoxP loci when they are located in the same direction on a DNA strand.

The purpose of this study was to construct a live gene deleted vaccine against pseudorabies virus with a newly isolated PRV using CRISPR/Cas9 and Cre/Lox systems.

MATERIALS AND METHODS

Ethics statement: Animal experiments were approved by Animal Ethics Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences and the Animal Ethics Committee approval number was Heilongjiang-SYXK 2015-005.

Isolation and identification of virulent PRV strain: A PRV variant was isolated and identified from a PRV Bartha-K61 vaccinated pig farm in Heilongjiang Province in China in 2017. The PRV isolate was cultured in PK15 cells and named Strain PRV-ZD. PRV-SC strain is the standard classical PRV virulent strain. PRV Bartha-K61 is the classical PRV attenuated vaccine.

Constructions of recombinant plasmid, preparation of virus genome and PCR amplification: Based on the PRV sequence (GenBank accession number: KJ789182.1), six pairs of primers were designed to amplify target genes and construct the vector. The guide RNA of TK/gE/gI genes of PRV was cloned into sgRNA / Cas9n expression vector (Addgene, Beijing, China) homologous arms of TK/gE/gI gene were amplified *in vitro* and LoxP, and Lox2272 were added, respectively. The expression frame containing eGFP, RFP, TK/gE/gI homologous arms was constructed by overlapping PCR using TK l-arm and TK r-arm (green), and gI/gE l-arm and gI/gE r-arm (red, two genes). The whole genome of PRV was extracted by TIANamp Virus DNA/RNA Kit (TIAGEN Biotech, Beijing, China). All the primer sequences are shown in Table 1. PRV gene knockout flowchart is presented in Fig. 1.

Cell culture, vector transfection and virus infection: As shown in Fig. 1, PK15 cells were co-transfected by Lipofectamine®2000 Kit (Thermo Fisher, Carsbad, CA) with recombinant plasmids, eGFP DNA fragment containing homologous arms of TK gene and RFP DNA fragment containing homologous arms of gI/gE gene. Transfected cells were inoculated with PRV-ZD at 8 hours after transfection. The infected cells were cultured in the DMEM solution containing 2% FBS.

Identification and purification of gene deletion virus: Cells containing two kinds of fluorescent proteins of PRV were selected by fluorescent microscopy and continuous plaque purification. The two kinds of fluorescent protein genes were knocked out by Cre/Lox system, and the cells containing PRV without fluorescence were obtained by low melting point agarose plaque purification to produce a triple gene-deleted live PRV strain, rZDΔTK-gE-gI. The genome was then extracted with TIANamp Virus DNA/RNA Kit (TIAGEN Biotech, Beijing, China), amplified by PCR and sequenced to identify the knockout effects of the three genes TK/gE/gI.

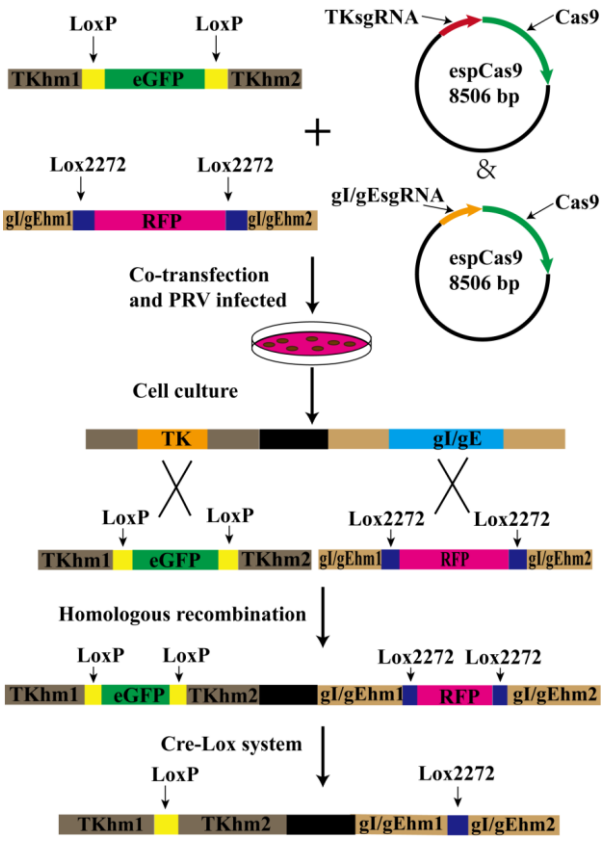

Fig. 1: Pseudorabies Gene Knockout Flow Chart. The homology arms containing TK gene, eGFP gene fragments on the LoxP site (green), homology arms containing the gE/gI gene, the RFP gene fragment at Lox2272 site (red) were constructed by the overlapping PCR method. These gene fragments and corresponding gene knock-out Cas9 recombinant plasmids were co-transfected into PK15 cells. Pseudorabies gene deletion strain was constructed by Cas9 gene editing system and homologous recombination technology, and fluorescent protein genes were introduced into the recombinant plasmid as a reporting system.

Table 1: Primer Sequences

Primer name	Primer Sequence	Length	Purpose
TK l-arm	: 5'-GGTGGCGCTGATCTCCGA-3'	1053 bp	Left homologous arm of
	R:5'ATAACTTCGTATAGCATACATTATACGAAGTTATCCCGGCGCGCTTCCGGGC-3'		TK gene
TK R-arm	F:5'ATAACTTCGTATAGCATACATTATACGAAGTTATATACCGGGAGAACCGGTCCG-3'	1137 bp	Right homologous arm of
	R: 5'-CGCGCGCATGAGCAGGCG-3'		TK gene
gl/gE l-arm	F: 5'-GGTGGCGCTGATCTCCGA-3'	1026 bp	Left homologous arm of
	R: 5'- ATTGAAGCATATTTCATAGGATATGCTTCAATA CGACGCCGGTACTGCGGA-3'		Gl/Ge gene
gl/gE r-arm	F: 5'- ATTGAAGCATATTTCATAGGATATGCTTCAATA ATACCGGGAGAACCGGTCCG-3'	1137 bp	Right homologous arm
	R: 5'-CGTCTGGGCGTGGGAGGCT-3'		of Gl/Ge gene
EGFP	F: 5'-ATGGTGAGCAAGGGCGAG-3'	720 bp	Fluorescent labeling
	R: 5'-TTACTTGTACAGCTCGTCC-3'		
DsRed	F: 5'-ATGGTGGCCTCCTCCGAG-3'	1395 bp	Fluorescent labeling
	R: 5'-CTACAGGAACAGGTGGTGG-3'		
TK-sgRNA	F: 5'-TCGTCTTTGACCGCCACC-3'	18 bp	TK gene knockout target
	R: 5'-GGTGGCGGTCAAAGACGA-3'		
gl/gE-sgRNA	F: 5'-CAGCGCCGTCCTCGTGCC-3'	18 bp	Gl/Ge gene knockout
	R: 5'-GGCACGAGGACGGCGCTG-3'		target

Sequencing analysis: PCR products were sequenced at Sangon Biotech (Shanghai, China) Co., Ltd. PRV sequence alignment was carried out using Bioedit software.

Determination of virus titer: PRV rZDΔTK-gE-gI was inoculated onto the PK15 cells and the virus titer was calculated by Reed-Muench method (Reed, 1938) .

Animal experiments: Thirty-five 3-week-old piglets were randomly divided into 7 groups (5 in each experimental group and 5 in the control group), including Bartha-K61/SC, Bartha-K61/PRV-ZD, rZDΔTK-gE-gI/SC, rZDΔTK-gE-gI/PRV-ZD, SC (mock/SC), PRV-ZD (mock/PRV-ZD) and the control group. Each piglet in the immunization groups was intramuscularly inoculated with 10^5 $TCID_{50}$ vaccine virus whereas each piglet in the control group was intramuscularly inoculated with PBS. Each piglet in Bartha-K61/PRV-ZD, rZDΔTK-gE-gI /PRV-ZD and mock/PRV-ZD was challenged with 2 ml of PRV-ZD containing 1.0×10^6 $TCID_{50}$ on the 14th day after immunization. In Bartha-K61/SC, rZDΔTK-gE-gI/SC and mock/SC piglets, 2 mL of PRV SC containing 1.0×10^5 $TCID_{50}$ was applied for challenge on the 14th day, and each one in the control group was inoculated with 2 ml of PBS. Serum samples and nasal and rectal swabs of piglets were collected for testing after challenge.

Serological tests and detection of virus shedding: All serum samples were detected by ELISA Kits for PRV gB-specific and gE-specific antibodies following the manufacturer′s instructions (IDEXX, Maine). The nasal and rectal swabs were treated and inoculated into PK15 cells to detect the virus titer.

Statistical analysis: The significant differences of the animal experiments were analyzed using GraphPad Prism (version 5.01) (San Diego, CA). Differences were considered statistically significant when $P<0.05$.

RESULTS

Isolation, culture and genome sequence analysis of PRV strain: There was an epidemic of pseudorabies in a PRV Bartha-K61 vaccinated pig farm in Heilongjiang Province in China in 2017, where samples were collected from the diseased animals. The PCR amplification results are shown in Fig. 2. A fragment of the same size as that of the positive control was amplified using the brain tissue, which was 865 bp (Fig 2A). The full-length PCR sequencing analysis of gE gene showed that the isolated strain was the similar to the pseudorabies variant after 2011 (Fig. 2B). The material that was tested PCR positive was inoculated into PK15 cells and cultured by successive passages. The isolate was named strain PRV-ZD.

Construction of a PRV rZDΔTK-gE-gI strain by knocking out three virulence related genes using CRISPR/Cas9 and Lox/Cre system: In order to knock out the virulence related genes (TK-gE-gI) of PRV efficiently and swiftly, CRISPR/Cas9 combined with Lox/Cre system was used to knock out three virulence genes at the same time. Meanwhile, two fluorescent proteins and continuous plaque purification were used to obtain the PRV strain with stable deletion of three genes (Fig. 3).

In terms of avoiding the false positive signal caused by the expression of fluorescent gene in the homologous recombinant donor template, the expression of GFP and RFP are driven by the endogenous virus promoter, which only occurs after the precise homologous recombination of DNA. In this study, GFP and RFP genes were designed to be located on both sides of LoxP and Lox2272 pairs, separately, to facilitate the removal of fluorescent marker genes.

TK, gE and gI are the main virulence-related genes of PRV and the main targets of PRV gene deficient vaccine. The simultaneous deletion of TK, gE and gI genes was realized by the homologous recombination mediated by CRISPR/Cas9 system. Firstly, sgRNA targeting TK, gE and gI genes (designed by web-based tools http://crispr.mit.edu/) were co-transfected into PK15 cells with DNA donors containing fluorescent selection genes and homologous arms. At 8 hours after transfection, the cells were infected with PRV-ZD with different MOI (infection complex) and incubated until the recombinant virus expressing red and green fluorescence was observed (Fig. 3A, 3B and 3C). It was noted that no green/red overlapping cells were observed in the control group (Fig. 3D).

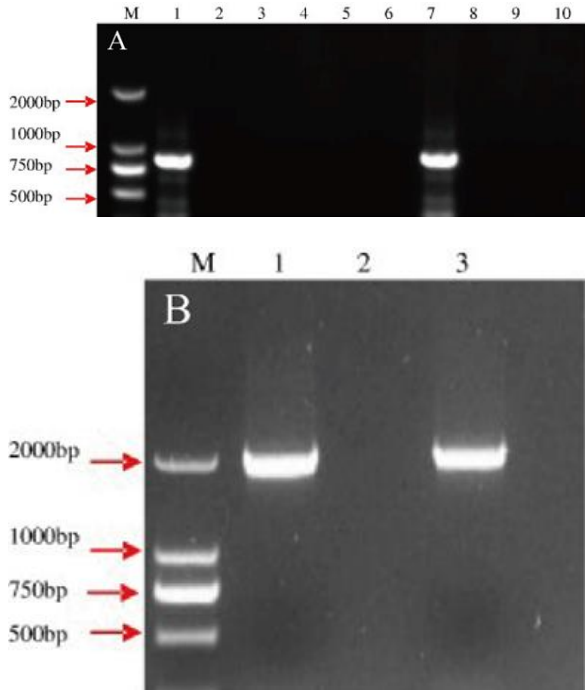

Fig. 2: Identification of PRV by PCR. A: Two of ten clinical samples were identified positive by PCR (the fragment size was 856 bp); B: gE gene PCR amplification results of two positive samples.

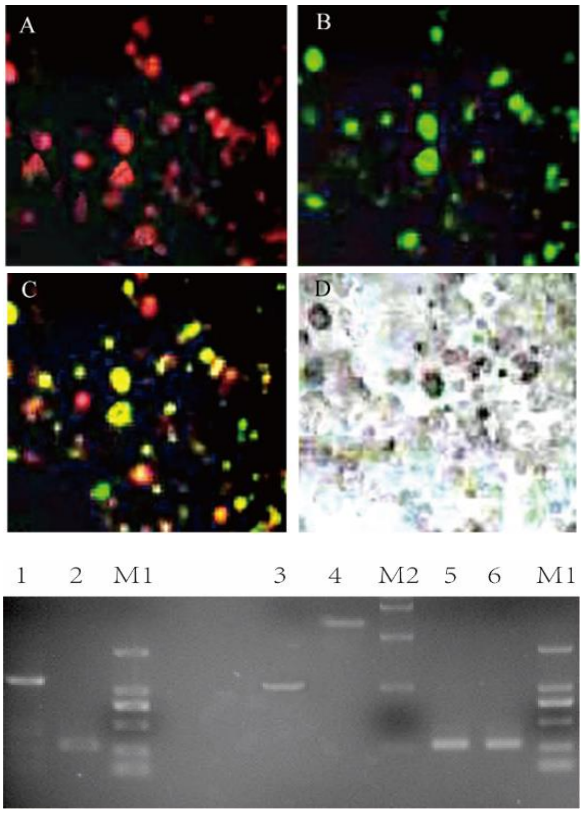

Fig. 3: Results of the three gene deletion PRV under the fluorescence microscope. A: Fluorescence result of PRV after replacement of TK gene by eGFP; B: Fluorescence result of PRV after replacement of gE/gI genes by RFP; C: Merged diagram of RFP and eGFP fluorescence observation results; D: Results in the same field of the fluorescence under light microscope; E: Identification results of the recombinant PRV by PCR. 1, 2: PCR results of PRV; M1: DL2000 Marker; M2: DL5000 Marker; 3: PCR results of TK gene of PRV in the control group; 4: PCR results of gE/gI genes of PRV in the control group; 5: PCR results of PRV with deletion of TK gene; 6: PCR results of PRV with deletion of gE/gI genes.

Plaques containing two kinds of fluorescence were selected for plaque purification to obtain pure recombinant virus (P2). The purity of the recombinant virus was verified by PCR amplification. As shown in Fig. 4, except for the control, positive control PCR bands were observed in all samples, indicating the integrity of virus DNA, while TK, gE and gI gene amplifications in the control group were still complete (Fig. 3E). Further experiments showed that the triple gene deletion virus was stable for six passages.

Observation of clinical symptoms after challenge: In order to evaluate the cross protection of the live vaccine against classical and variant PRV strains, a commercial PRV live vaccine (Bartha-K61) and PRV rZDΔTK-gE-gI were used to inoculate piglets. PRV-SC and PRV-ZD were then used to challenge piglets and the rectal temperature, clinical signs, weight gain and virus titers in the blood of vaccinated piglets 14 days after vaccination were checked. As shown in Fig. 4A, the body temperature of mock/SC group and mock/PRV-ZD group increased to 40.3-41.8°C on the second day after challenge. Compared with the mock/SC group, the average temperature of mock/PRV-ZD group was higher. It also revealed obvious clinical symptoms of PR, mental depression, respiratory distress, loss of appetite, vomiting, tremor and ataxia, and finally all died within 7 days. All the piglets challenged with PRV-ZD died between 4 and 7 days. All the non-immunized piglets in the control group challenged with PRV-SC died between 6 and 8 days (Fig. 4B). These results showed that PRV-ZD had higher virulence in 4-week-old piglets. Yet, all piglets in rZDΔTK-gE-gI /PRV-ZD, rZDΔTK-gE-gI/SC and Bartha-K61/SC groups remained healthy like those in the control group and no PR symptoms were detected during the 2-week observation period.

Vaccination can significantly shorten the growth stagnation period and decrease the economic loss caused by PRV infection. Thus, we evaluated the effect of the live vaccine on the weight gain of Bartha-K61- and rZDΔTK-gE-gI-immunized piglets after challenge with virulent virus. As shown in Fig. 4C, the weight gains in all the groups were significantly lower than that of the control group ($P<0.05$). Pigs in rZDΔTK-gE-gI/SC and Bartha-K61/SC groups gained similar weight throughout the experiment ($P>0.05$). However, the body weight of rZDΔTK-gE-gI /PRV-ZD group was significantly higher than that of Bartha-K61/PRV-ZD group ($P<0.05$).

Virus detection: From day 0 to day 14, nasal and anal swabs were collected from piglets in each group to determine the virus shedding of the experimental animals. The results showed that virus shedding was detected in all piglets, including the controls and the challenged animals. In the four immunization groups, virus shedding started on the second day and lasted for only 4 days, which was shorter than that of the non-immunized piglets, as shown in Fig. 4D. Vaccination of piglets with Bartha-K61 vaccine reduced the viral load after challenge with PRV-ZD. From the second day to the seventh day, the virus shedding content of rZDΔTK-gE-gI/PRV-ZD group was significantly lower than that of Bartha-K61/PRV-ZD group ($P<0.05$). Consistent results were observed between PRV-ZD/SC and Bartha-K61/SC groups.

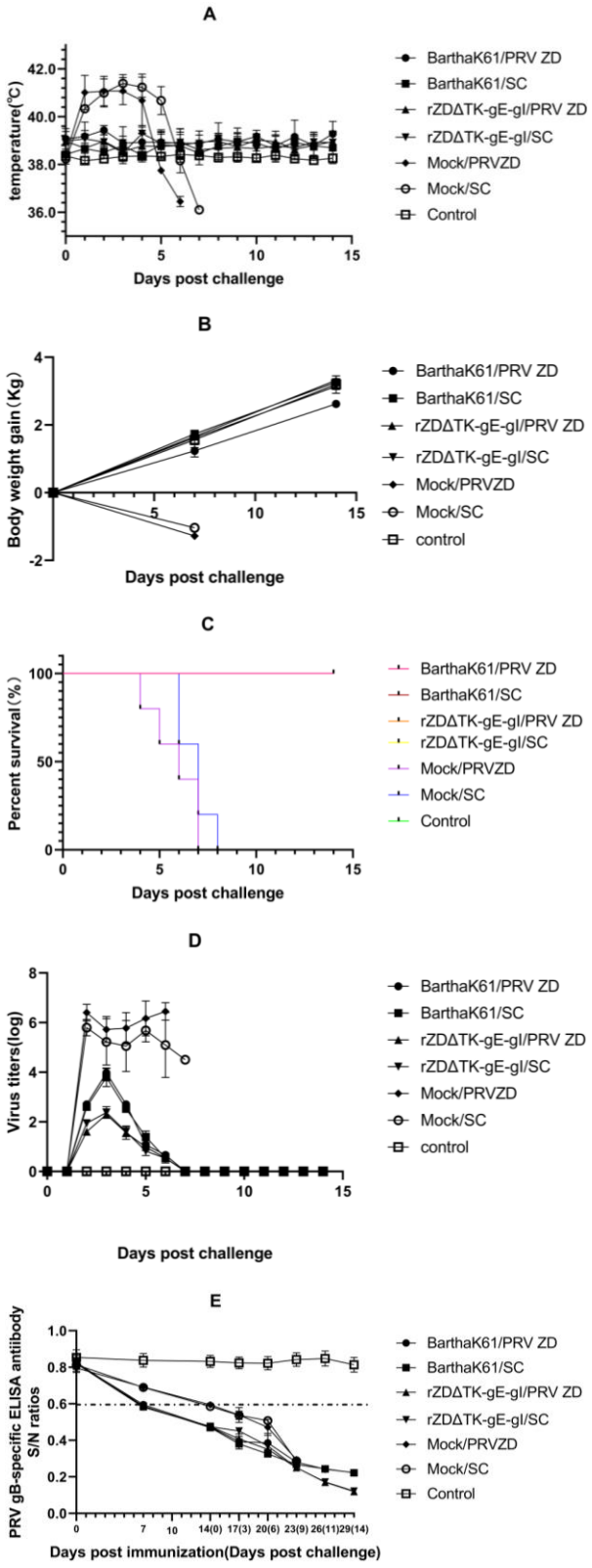

Fig. 4: Results of piglet experiments. Piglets challenged with PRV-ZD: A. Temperature; B: Survival rates; C: Body weights; D: Virus titers by PCR in the nasal and anal swabs; E: gB-specific ELISA antibody S/N ratios in vaccinated piglets at 0, 3, 6, 9, 11 and 14 days after immunization with rZDΔTK-gE-gI or Bartha-K61.

Antibody response to PRV infection: In order to monitor the levels of PRV-gB specific antibody produced by PRV-ZD and SC strains, serum samples were collected after vaccination and ELISA used to detect the antibodies. The results showed that the piglets immunized with rZDΔTK-gE-gI or Bartha-K61 had the gB-specific antibody on the 3rd day, and the antibody levels of gB increased steadily in all the immunized pigs, and at the 15th day all the infected piglets produced the high level of anti-gB antibody. During the 14-day after immunization, no specific antibody of gB was detected in the control group (Fig. 4E). These results showed that rZDΔTK-gE-gI vaccine candidate could protect pigs from classical PRV and PRV variants.

DISCUSSION

PR has always been regarded as one of the most imperative infectious diseases in pig industry (Liu *et al.*, 2018) . Vaccinations with large doses of Bartha-K61 vaccine were an effective method to control the prevalence and spread of PR (Zhou *et al*., 2017) . This method was very effective, and only a few cases of pseudorabies had been reported. Since 2011, the PRV epidemic successively broke out in a large number of pig farms in many provinces in China, including the large-scale pig farms where pigs were immunized with attenuated PRV vaccine, causing huge economic losses. The main manifestations of the infected animals were that the positive rate of gE antibody in the pig population significantly increased, the sows had abortion, weak piglets and stillborn fetuses, and newborn piglets had obvious neurological symptoms and died in a large number. All the piglets in these pig farms were vaccinated with the attenuated PRV gE gene deletion vaccine. PRV could be isolated from dead piglets and aborted fetuses. The preliminary results showed that compared with the previous PRV strains, the new PRV strains had significant variations, and the pathogenicity of the new PRV strains was significantly increased. The existing vaccine cannot effectively protect against the current epidemic strains. Therefore, it is an urgent task to develop a new PR vaccine against the variant.

With the advance of molecular biology and gene engineering technology, the molecular biological characteristics and gene functions of PRV have been clearly recognized. The absence of one or more non-essential genes for PRV replication can greatly reduce the virulence of PRV, but at the same time does not affect its proliferation and immunogenicity. Many research results show that PRV gene deletion vaccines have high safety and good immunogenicity and have been widely used in practical production. A TK/gE/gI triple gene-deleted PRV derived from a PRV variant was generated by using bacterial artificial chromosome techniques, which elicited high levels of protective gB-specific antibodies and provided full protection to the viral challenge after vaccination (Zhang *et el.*, 2015). A TK/gE/gI-deleted PRV rZJ01ΔTK/gE/gI was constructed from the gE/gI-deleted PRV, and rZJ01ΔTK/gE/gI provided full protection against a lethal challenge with the PRV ZJ01 variant in pigs and had lower pathogenicity compared with the gE/gI gene-deleted virus strain rZJ01ΔgE/gI (Dong *et al.*, 2017).

PRV variant PRV-ZD was isolated from a pig farm where piglets were inoculated with Bartha-K61 vaccine in Heilongjiang Province of China in 2017. Compared with SC strain, PRV-ZD strain had stronger pathogenicity to

piglets, and the lethal rate to 3-week-old piglets was 100%. Given the above results, we combined CRISPR/Cas9 system with high gene editing efficiency to knock out the virulence related genes of PRV variant and used mature Cre/Lox system to remove the marker genes in gene editing to obtain stable PRV triple gene deletion attenuated strain rZDΔTK-gE-gI without foreign genes.

When PRV infects blood monocytes, monocytes express glycoproteins recognized by specific antibodies on their plasma membrane, thus initiating antibody dependent complement pathway and resulting in cell lysis and virus clearance. Nevertheless, gE/gI complex has the ability to bind to IgG, which prevents complement pathway and leads to immunosuppression of the body. Thus, the virus can reproduce in a great number in the body and further damage the body. TK gene deletion vaccine was also the earliest gene engineered vaccine. The growth of PRV with TK gene deletion increased to a good level in the cell cultures like the wild strain, but its virulence and latent infection ability were greatly reduced. In this study, the TK/gE/gI genes were knocked out to avoid the immunosuppression of the body, and the constructed attenuated vaccine with triple gene deletions proliferated well on PK15 cells.

Conclusions: In conclusion, a TK/gE/gI triple gene-deleted PRV was successfully constructed using CRISPR/Cas9 and Cre/Lox system-based strategy and this recombinant vaccine candidate provided full protection against a PRV variant in 3-week-old piglets, which could be used as an ideal vaccine candidate for preventing pigs from PRV variant infection in China.

Authors contribution: MW, LW, and QZ designed the study. MW executed the experiments. All authors analyzed and interpreted the data and approved the final version of the manuscript.

REFERENCES

Cong X, Lei JL, Xia SL, *et al*., 2016. Pathogenicity and immunogenicity of a gE/gI/TK gene-deleted pseudorabies virus variant in susceptible animals. Vet Microbiol 182:170-7.

Dong J, Bai J, Sun *et al*., 2017. Comparative pathogenicity and immunogenicity of triple and double gene-deletion pseudorabies virus vaccine candidates. Res Vet Sci 115:17-23.

Fan J, Zeng X, Zhang G, *et al*., 2016. Molecular characterization and phylogenetic analysis of pseudorabies virus variants isolated from Guangdong province of southern China during 2013-2014. J Vet Sci 17:369-75.

Van Duyne GD, 2014. Cre Recombinase. Microbiol spectr 3:MDNA3-0014-2014.

Hu RM, Zhou Q, Song WB, *et al*., 2015. Novel pseudorabies virus variant with defects in TK, gE and gI protects growing pigs against lethal challenge. Vaccine 33:5733-40.

Liu Y, Zhang S, Xu Q, *et al*., 2018. Investigation on pseudorabies prevalence in Chinese swine breeding farms in 2013-2016. Trop Anim Health Prod 50:1279-85.

Ma Y, Zhang L and Huang X, 2014. Genome modification by CRISPR/Cas9. FEBS J 281:5186-93.

Masot AJ, Gil M, Risco D, *et al*., 2017. Pseudorabies virus infection (Aujeszky's disease) in an Iberian lynx (Lynx pardinus) in Spain: a case report. BMC Vet Res 13:6.

Papageorgiou KV, Suarez NM, Wilkie GS, *et al*., 2016 Genome Sequences of Two Pseudorabies Virus Strains Isolated in Greece. Genome Announc 4:1.

Reed LJ, 1938. A simple method of estimating fifty percent endpoints. Am J Hyg 27.

Ren M, Lin H, Chen S, *et al*., 2018. Detection of pseudorabies virus by duplex droplet digital PCR assay. J Vet Diagn Invest 30:105-12.

Sun Y, Liang W, Liu Q, *et al*., 2018. Epidemiological and genetic characteristics of swine pseudorabies virus in mainland China between 2012 and 2017. Peer J 6:e5785.

Tong W, Liu F, Zheng H, *et al*., 2015. Emergence of a Pseudorabies virus variant with increased virulence to piglets. Vet Microbiol 181:236-40.

Wang T, Xiao Y, Yang Q, *et al.,* 2015. Construction of a gE-Deleted Pseudorabies Virus and Its Efficacy to the New-Emerging Variant PRV Challenge in the Form of Killed Vaccine. Biomed Res Int 2015:684945.

Wu Q, Zhang H, Dong H, *et al*., 2018. Seroprevalence and risk factors associated with Pseudorabies virus infection in Tibetan pigs in Tibet. BMC Vet Res 14:25.

Yang QY, Sun Z, Tan FF, *et al*., 2016. Pathogenicity of a currently circulating Chinese variant pseudorabies virus in pigs. World J Virol 5:23-30.

Yu T, Chen F, Ku X, *et al*., 2016. Complete Genome Sequence of Novel Pseudorabies Virus Strain HNB Isolated in China. Genome Announc 4.

Zhang C, Guo L, Jia X, *et al*., 2015. Construction of a triple genedeleted Chinese Pseudorabies virus variant and its efficacy study as a vaccine candidate on suckling piglets. Vaccine 33:2432-7.

Zhou J, Li S, Wang X, *et al*., 2017. Bartha-k61 vaccine protects growing pigs against challenge with an emerging variant pseudorabies virus. Vaccine 35:1161-6.