Construction and immunological evaluation of recombinant Newcastle disease virus vaccines expressing highly pathogenic porcine reproductive and respiratory syndrome virus GP3/GP5 proteins in pigs

He Zhang[a], Fulong Nan[a,b], Zhuoxin Li[a,c], Guanyu Zhao[a,b], Changzhan Xie[a,c], Zhuo Ha[a,d], Jinyong Zhang[a,c], Jicheng Han[a,e], Pengpeng Xiao[a,f], Xinyu Zhuang[a], Wei Wang[a,g], Jinying Ge[h], Mingyao Tian[a], Huijun Lu[a,*], Zhigao Bu[h,**], Ningyi Jin[a,i,*]

[a] Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Institute of Military Veterinary Medicine, Academy of Military Medical Sciences, Academy of Military Sciences, Changchun, China
[b] College of Veterinary Medicine, Jilin University, Changchun, China
[c] College of Animal Science and Technology, Jilin Agricultural University, Changchun, China
[d] College of Veterinary Medicine, Northeast Agricultural University, Harbin, China
[e] Medical College, Yanbian University, Yanji, China
[f] Institute of Virology, Wenzhou University, Wenzhou, China
[g] College of Animal Science and Technology, Guangxi University, Nanning, China
[h] National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China
[i] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China

ARTICLE INFO

ABSTRACT

Highly pathogenic porcine reproductive and respiratory syndrome (HP-PRRS) poses a significant threat to the pig industry, for which vaccination is considered to be an effective means of prevention and control. Here, we developed two recombinant Newcastle disease virus (NDV) LaSota-vectored PRRS candidate vaccines, rLaSota-GP5 and rLaSota-GP3-GP5, using reverse genetic techniques. The two recombinant viruses exhibited a high degree of genetic stability after 10 successive generations in chicken embryos. There was no significant difference in pathogenicity compared with the rLaSota parent strain in poultry, mice and pigs. The recombinant viruses could not be detected in the feeding environment of immunized pigs, but could be detected in the organs and tissues of pigs for no more than 10 days after immunization. Importantly, in contrast to rLaSota-GP5, rLaSota-GP3-GP5 elicited both significant humoral and cellular immune responses in pigs. In particular, the neutralizing antibody titer in the rLaSota-GP3-GP5 group was 1.51 times significantly higher than that of the commercial vaccine group at 42 days post-immunization. At the same time, there was significant difference in the level of IFN-γ between the rLaSota-GP3-GP5 group and the commercial vaccine group. Furthermore, the viral load in the organs and tissues of rLaSota-GP3-GP5-immunized pigs was substantially lower than that of unimmunized pigs after being challenged with HP-PRRS virus GD strain. These results suggest that rLaSota-GP3-GP5 is a safe and promising candidate vaccine, and there is potential for further development of a recombinant virus vaccine for PRRS using NDV.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first confirmed in China in 1996, and has since become one of the most challenging diseases that threaten the pig industry due to its capacity for wide dissemination and high genetic diversity (Brito et al., 2014; Zhou and Yang, 2010). During the first half of 2006, several pig farms in South China experienced a highly pathogenic infectious disease with typical symptoms of prolonged high fever and a strong ability to spread quickly. It was subsequently confirmed using pathogen isolation and

genomic evaluation and analysis that the highly pathogenic PRRS virus (HP-PRRSV; North American type) was the cause of the outbreak (An et al., 2011; Feng et al., 2008; Tian et al., 2007). Recently, HP-PRRS has been reported in almost all regions of China, except Tibet. Moreover, HP-PRRSV strains remain the most pathogenic ones among the endemic strains of PRRS in China, resulting in huge economic losses to the pig industry (Huang et al., 2019; Liu et al., 2015).

PRRSV is a member of the family *Arteriviridae* of the order *Nidovirales*, and is an enveloped, positive-sense and single stranded RNA virus with a genome consisting of approximately 15 kb encoding at least 10 overlapping open reading frames (ORFs) (Kimpston-Burkgren et al., 2017; Snijder et al., 2013). The viral GP5 protein is encoded by ORF5 (approximately 30 kDa) and constitutes one of the most important structural proteins of PRRSV, with highly conserved glycosylation sites and hypervariable regions on both sides of its structural domain (Chen et al., 2016). Moreover, GP5 displays strong immunogenicity and can induce the production of neutralizing antibodies (Ostrowski et al., 2002; Wang et al., 2009), as well as cellular immune responses. Thus, GP5 has become a major target protein in PRRSV vaccine development. The viral GP3 protein encoded by ORF3 is the most glycosylated PRRSV protein with seven putative N-linked glycosylation sites that are well-conserved among different PRRSV strains (Gonin et al., 1998). Like GP5, GP3 is also highly antigenic and plays an important role in the clearance of the viral infection and in the protection of piglets against PRRSV (Chen et al., 2014; Katz et al., 1995).

As a member of the *Paramyxoviridae* family, Newcastle disease virus (NDV) has been widely developed as a vaccine carrier for the prevention of human and animal diseases using reverse genetic technology (DiNapoli et al., 2010; Khattar et al., 2010; Kim and Samal, 2016; Kortekaas et al., 2010). The replication ability of NDV in non-avian hosts was seriously affected, but the possibility of NDV replication still exists in pigs (Bukreyev et al., 2005; Nakaya et al., 2001; Shen et al., 2006). Furthermore, NDV can not only induce humoral, cellular and mucosal immune responses, but stimulate the production of a large number of interferons in vivo (Bu et al., 2016). In the present study, two recombinant NDV LaSota strains expressing the HP-PRRSV GP5 or GP3-GP5 proteins were constructed. The biological characteristics and immunogenicity of these recombinant viruses and vaccine efficacy in pigs were evaluated.

2. Materials and methods

2.1. Viruses, cells, and experimental animals

BHK-21 cells and MARC-145 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin (10,000 U/mL)-streptomycin (10,000 μg/mL). The North American-type HP-PRRSV GD strain was provided by South China Agriculture University. The NDV modified attenuated strain of rLaSota was propagated in the allantoic cavity of nine-day-old Specific Pathogen Free (SPF) chicken embryonated eggs (purchased in Harbin Weike Biotechnology Development Company, China).

SPF chickens were bred and housed in negative pressure isolators at the Animal Facility of Harbin Veterinary Research Institute. Four-week-old female BALB/c mice were purchased from Changchun Institute of Biological Products and housed in the Experimental Animal Room of Academy of Military Medical Sciences. Forty-nine crossbred F1 (Landrace × York) piglets (weaned at 28 days old) seronegative for PRRSV and PCV2 were obtained from a certified PRRS-free farm in Changchun (Jilin, China). The piglets were then randomly divided into nine groups (n = 5 / 6), which were housed separately.

2.2. Plasmid construction and recombinant virus rescue

The ORF5 and ORF3 genes of the PRRSV GD strain (GenBank:

EU825724.1) were amplified by reverse transcription-polymerase chain reaction (RT-PCR). The recombinant plasmids expressing exogenous genes were constructed as previously described (Ge et al., 2007), except that ligation was performed using the ClonExpress MultiS One Step Cloning Kit (Vazyme, China). Primers would be made available upon request. In particular, an IRES sequence was inserted between the ORF3 and ORF5 genes for the PBRN-PL-ORF3-ORF5. In addition, two recombinant viruses, rLaSota-GP5 and rLaSota-GP3-GP5, were rescued as described previously.

2.3. Identification and stability of the recombinant viruses

2.3.1. Sequence analysis by RT-PCR

RNA was extracted from the recombinant viruses and reverse transcribed to cDNA to be used as PCR templates. GP5 and GP3-GP5 fragments were accurately amplified by an additional sequencing range of 802 bp.

2.3.2. Protein expression analysis by Western blot and immunofluorescence assay

A monolayer of BHK-21 cells was infected with rLaSota or recombinant viruses at a multiplicity of infection (MOI) of 1. After 48 h, the total cellular proteins were extracted using RIPA lysis buffer (Solarbio, China) with 1% PMSF on ice for 35 min. Western blot was performed as described previously (Shen et al., 2007), except the primary antibodies of anti-HP-PRRSV serum from pigs and anti-NDV serum from chickens. The protein bands were visualized under a luminescent image analyzer (GE Amersham Imager 600, USA) using an ECL chemiluminescent substrate reagent kit (Thermo Scientific, USA).

After 10 consecutive generations, different passages were selected to assess the virus stability using an immunofluorescence assay (IFA). BHK-21 cells were grown in 24-well plates and infected with either rLaSota or the recombinant viruses. At 24 h post-infection, the cells were fixed in a 4% paraformaldehyde fix solution (Beyotime Biotechnology, China) for 30 min at room temperature and then washed three times with PBS. Anti-HP-PRRSV serum from pigs or anti-NDV serum from chickens was used as the primary antibody and FITC-conjugated rabbit anti-pig (Bioss antibodies, China) or FITC-conjugated goat anti-chicken antibody (Abcam, UK) was used as the secondary antibody. The cells were observed under a fluorescence microscope.

2.4. Assessment of recombinant virus pathogenicity in poultry, mice and pigs

Chicken embryo allantoic fluid was collected at 24, 48, 72, 96, and 120 h post-inoculation to determine the growth curve of the recombinant viruses. Based on the diagnostic methods recommended by the Office International Des Epizooties (OIE, 2012), the intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI), and mean death time (MDT) were determined in SPF chicken embryos or SPF chickens. A definitive assessment of viral virulence was based on an intracerebral pathogenicity test by international agreement. The most virulent viruses will yield indices that approach the maximum score of 2.0, whereas attenuated strains will give rise to values close to 0.0.

Eighty four-week-old female BALB/c mice were randomly divided evenly into eight groups to assess the pathogenicity of recombinant viruses in mice. After an intramuscular (i.m.) and intracerebral inoculation (i.c.) with $2 \times 10^{7.0}$ 50% egg infective dose (EID_{50}) /mL of the viruses in a volume of 0.1 or 0.03 mL, all mice were observed for weight loss or death twice daily for 14 days. At 14 dpi, heart, liver, spleen, lung, kidney and or brain tissues were collected to analyze the virus residue by PCR.

Twenty-four four-week weaned piglets were randomly divided evenly into four groups to evaluate the safety of recombinant viruses and virus replication ability in vivo. rLaSota-GP3-GP5, rLaSota-GP5 and rLaSota were immunized by intramuscular injection at a dose of $2 \times 10^{8.0}$ EID_{50}/mL in 2 mL, respectively. PBS was used as the negative

control group at a dose of 2 mL in the same manner (Grouping details: Supplementary Table 1). One pig was randomly euthanized at 0, 3, 7, 10, 14, and 21 days post-immunization (dpi) and tissues and organs of the heart, liver, spleen, lung, kidney, brain, intestine, blood, muscle at injection site, and lymph nodes were collected and analyzed by quantitative real-time PCR. The partial fragments of NDV NP gene were amplified using the GoTaq® qPCR Master Mix kit (Promega, USA). Meanwhile, immunohistochemistry for NDV was performed on sections of formalin-fixed paraffin-embedded tissues involving the heart, liver, spleen, lung, kidney, brain, intestine, and lymph nodes. The lungs of chickens infected with NDV were used as positive controls. All sections were incubated with primary antibody (anti-NDV serum from rabbit) and secondary antibody (Goat anti-rabbit IgG/HRP; Solarbio, China), respectively. DAB (Gene Tech, China) was used as the chromogen, and all sections were counterstained with hematoxylin. Genome extraction kit (TIANGEN, China) was used to extract the pig peripheral blood genome to find out whether the gene recombination with NDV occurred by PCR. Samples of pig feces, urine, water and feed in the surrounding environment were collected to detect virus efflux.

2.5. Immunization and challenge in pigs

Twenty-five four-week-old piglets were randomly divided evenly into five groups. Animals in groups 1 and 2 were intramuscularly vaccinated with rLaSota-GP5 and rLaSota-GP3-GP5, respectively at a dose of $2 \times 10^{8.0}$ EID$_{50}$/mL in 2 mL. As control groups, groups 3 — 5 were vaccinated with rLaSota ($2 \times 10^{8.0}$ EID$_{50}$/mL), commercial vaccine ($1 \times 10^{5.0}$ TCID$_{50}$/mL, Live CH-1R Strain; Shanghai HILE, China), or PBS, respectively at the same dose and in the same manner (Grouping details: Supplementary Table 2). Four weeks after the initial immunization, all pigs except the commercial vaccine group received a boost immunization via the same route.

At 42 dpi, all groups were challenged with 3 mL of the HP-PRRS virus GD strain ($1 \times 10^{6.0}$ TCID$_{50}$/mL, i.m.).

2.6. Evaluation of serum antibody levels

At 0, 7, 14, 21, 28, 35, and 42 dpi, serum samples collected from all experimental pigs were analyzed for specific antibody titers as described previously (Ren et al., 2014), except for the purified GP5 proteins of the North American-type PRRSV as ELISA coated antibody. Moreover, neutralizing antibody titers were determined using a modified neutralization (NT) test as described previously (Takikawa et al., 1997), except for virus of the HP-PRRSV GD strain (200 TCID$_{50}$). NT titers of 2 or higher were considered to be positive.

2.7. Cytokine levels analysis

Commercial swine cytokine ELISA kits were used to analyze the concentration of IFN-γ (Sigma, USA) and IL-4 (TSZ, USA). Serum samples collected from the pigs at 14 dpi and 42 dpi were determined in accordance with the manufacturer's protocol.

2.8. Isolation of peripheral blood lymphocytes and proliferation assay

At 42 dpi, peripheral blood was collected from all the pigs into anticoagulative tubes containing sodium citrate. Isolation of the lymphocytes was performed as described previously (Han et al., 2018). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS in an atmosphere containing 5% CO$_2$ at 37 °C. A density of 1×10^5 lymphocytes was plated into each well of a 96-well tissue culture plate. Each sample was tested in three replicate wells. The HP-PRRSV GD strain (MOI = 1; 50 μL/well) was co-cultured with the lymphocytes as the specific antigen under the culture conditions described above. Concanavalin A (Con A; 10 μg/mL, Sigma; 50 μL/well) and medium alone (50 μL/well) were respectively co-cultured with the lymphocytes

as the positive and negative control, respectively. After 72 h, Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan; 10 μL/well) was added to each well and incubated for 4 h. The absorbance values of the samples were measured at 450 nm using a microplate reader. The stimulation index (SI) was used to represent the results of the proliferation assay, which was defined as the ratio of the mean absorbance value of the stimulators to that of the negative control.

2.9. Measurement of body temperature and organ viral load

Following viral challenge, clinical signs and body temperature were monitored daily in each of the pigs. Two weeks later, the pigs were euthanized and a pathological examination was performed. For further viral detection, the blood, heart, spleen, lung, and kidney samples were collected. RNA was extracted from the samples as described above. The partial fragments of ORF7 gene of the HP-PRRSV GD strain were amplified using the GoTaq® qPCR Master Mix kit (Promega, USA) to determine the viral load in the organs.

2.10. Statistical analysis

Data on virus titers, safety evaluation and vaccine efficacy experiments was analyzed with GraphPad Prism software 7.0 (San Diego, CA, USA). The levels of cytokines, SI values and viral loads in different groups were determined by applying One-way repeated measurement ANOVA and Least significance difference (LSD). P value was used to describe significant statistical differences. Specifically, $P \leq 0.01$, highly significant; $0.01 < P \leq 0.05$, significant; $P > 0.05$, not significant. Data is presented as the mean ± standard deviation (SD).

3. Results

3.1. Generation and identification of two recombinant viruses expressing exogenous genes

Based on the NDV reverse genetic system established by Ge et al. (Ge et al., 2007), either the ORF5 genes or ORF3-ORF5 genes from HP-PRRSV were cloned into the NDV genome at the *Pme* I restriction site between the P and M gene using homologous recombination (Fig. 1A). The recombinant viruses were rescued by plasmid transfection and chicken embryo inoculation. After 72 h, allantoic fluid was collected and the titer for both reached $10^{8.7}$ EID$_{50}$/mL. The presence of the exogenous genes was identified by RT-PCR (data not shown).

To confirm the expression of GP3 and GP5, Western blot was performed 48 h after viral infection at an MOI of 1. As expected, the protein collected from BHK-21 cells infected with the recombinant viruses displayed an effective reaction with pig serum against HP-PRRSV and chicken serum against NDV. However, the protein collected from BHK-21 cells infected with rLaSota reacted effectively with chicken serum against NDV only, but produced a noneffective reaction with pig serum against HP-PRRSV (data not shown). In addition, the two recombinant viruses were characterized by a high degree of growth stability between different generations (IFA results not shown). These results indicate that the exogenous protein was correctly expressed in the recombinant viruses.

3.2. Evaluation of recombinant virus virulence in chicken embryos, poultry, mice, and pigs

The growth kinetics of rLaSota-GP5 and rLaSota-GP3-GP5 were similar to those of rLaSota, and the peak value (recombinant viruses: 8.7 logEID$_{50}$/mL; rLaSota: 9.3 logEID$_{50}$/mL) was reached for both at 72 h (Fig. 1B).

In this study, the MDT, ICPI, and IVPI values of rLaSota-GP5 and rLaSota-GP3-GP5 were consistent with those of the rLaSota strain (Fig. 1C). Importantly, the ICPI values of the three tested viruses were
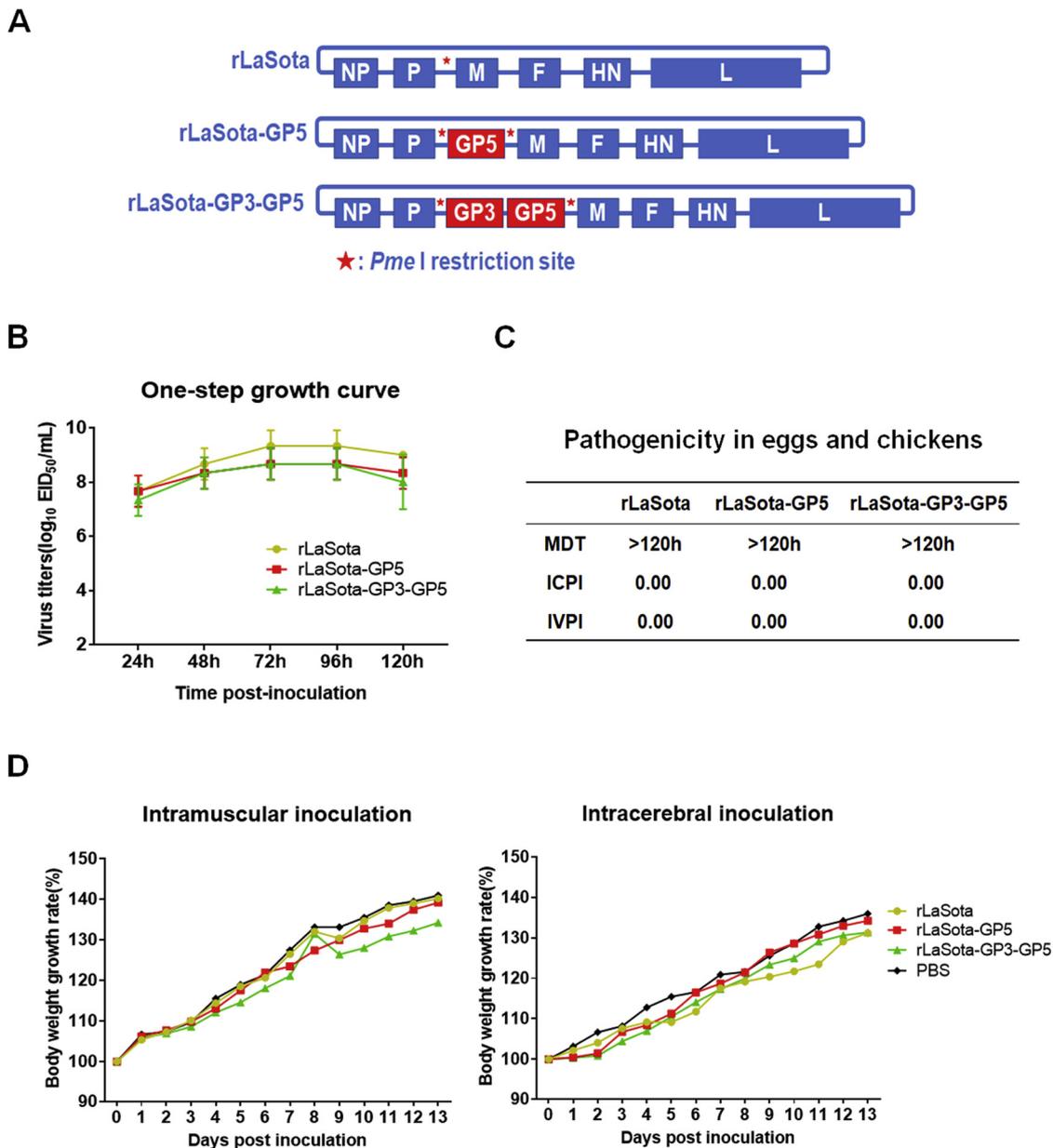
Fig. 1. Generation and virulence evaluation of two recombinant viruses. HP-PRRSV GP5 / GP3-GP5 genes was inserted between P and M genes of NDV vector by the Pme I restriction site (A). Chicken embryo allantoic fluid was collected at different time points to draw one-step growth curves of recombinant viruses (B). Mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) values of recombinant viruses were determined in SPF eggs and chickens (C). Weight changes after intramuscular or intracerebral inoculation of recombinant viruses in mice were measured for two consecutive weeks (D).

all 0.0, which demonstrated that the recombinant viruses were both attenuated strains.

To further confirm the safety of the recombinant viruses in mammals, intracranial and intramuscular injections were performed in mice. No abnormal changes in diet or activity were observed in any of the mice two weeks after the injection. The body weight of the animals who received either rLaSota-GP5 or rLaSota-GP3-GP5 changed in a similar manner to those that received rLaSota (Fig. 1D). In addition, virus was not detected in the heart, liver, spleen, lung, kidney or brain tissues that were collected at 14 dpi and analyzed by PCR, suggesting that the recombinant viruses did not induce adverse growth inhibition in mice. These results indicate that the insertion of the exogenous genes did not change the virulence and pathogenicity of NDV.

Safety and replication ability of recombinant viruses in pigs were important indicators for vaccine evaluation. Organs and tissues were collected at 0, 3, 7, 10, 14, and 21 dpi, and were evaluated at gene and protein levels, respectively. The presence of NDV vector gene could be detected only at 3 and 7 dpi by quantitative real-time PCR in the heart, liver, spleen, lung, kidney, brain, lymph node, blood and muscle. From the tenth day, NDV gene could not be detected in any of the organs and tissues of the four groups (Fig. 2A). There was the possibility that NDV was replicated in pigs within at least 7 days after immunization. Nevertheless, compared with the positive control group, the immunohistochemical results of all the four groups were negative within 21 days (Fig. 2B). Besides, no virus was detected in feces, urine, water or feed in the surrounding environment. Meanwhile, there was no gene integration between the recombinant viruses and pig genome (Fig. 2C). No obvious lesions were found in the immunization and control groups (pictures not showed). These results indicate that the recombinant viruses had limited replication capacity in pigs with good safety.
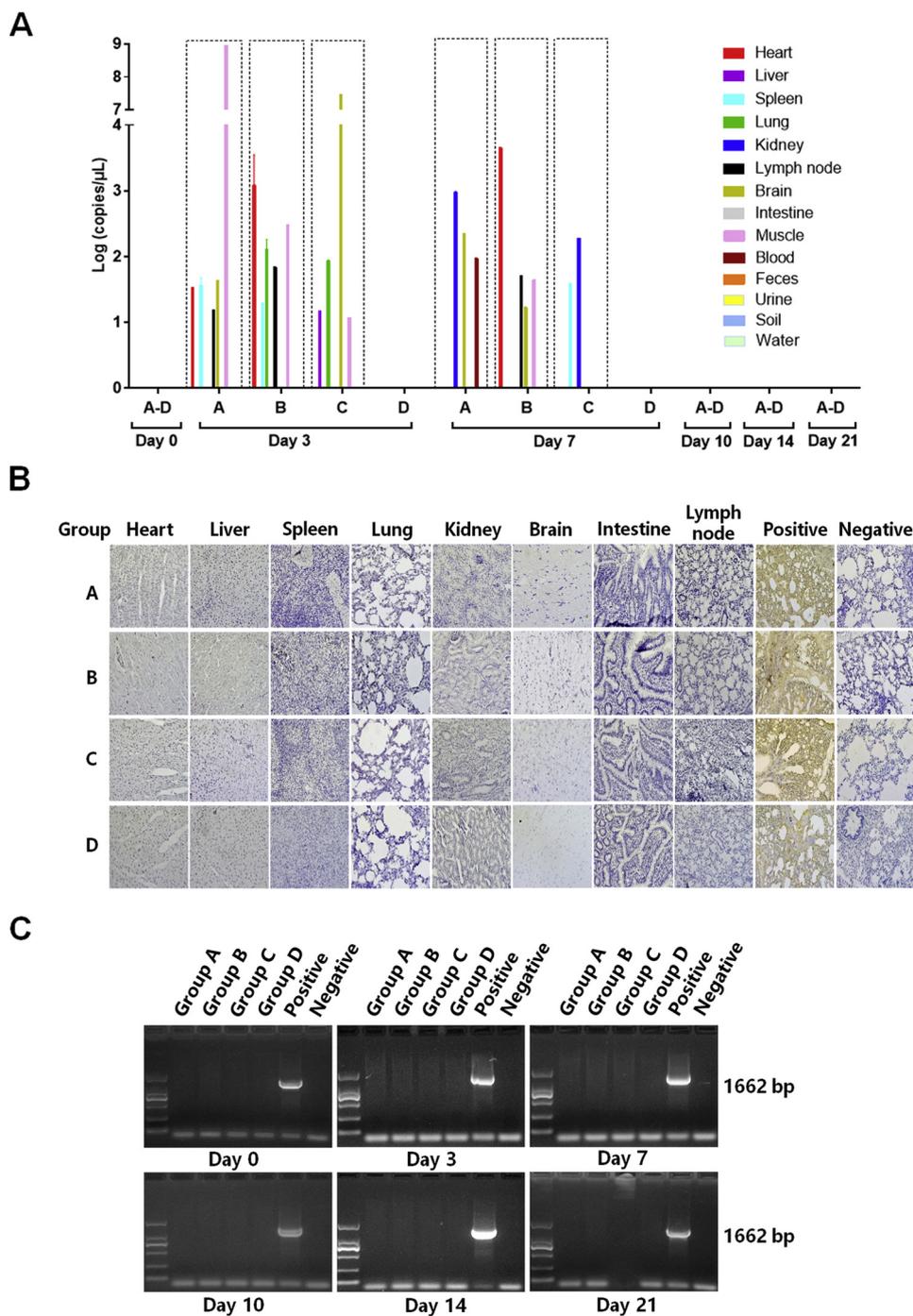
Fig. 2. Evaluation of safety and replication ability of two recombinant viruses in pigs. Tissues and organs samples of pigs were collected at 0, 3, 7, 10, 14, and 21 dpi (Group A: rLaSota-GP3-GP5; Group B: rLaSota-GP5; Group C: rLaSota; Group D: PBS). Viral load was analyzed by quantitative real-time PCR (A). Immunohistochemistry was performed as described in materials and methods and the representative results at 7 dpi were showed (B). The integration of recombinant viruses and pig genome was identified by amplification of NDV-specific gene fragments (C).

3.3. Recombinant virus rLaSota-GP3-GP5 induced a greater antibody response in pigs than rLaSota-GP5

According to the immunization strategy (Fig. 3A), serum samples from all of the pigs were collected and separated at 0, 7, 14, 21, 28, 35, and 42 dpi for the determination of PRRSV-specific neutralizing antibodies.

After the initial immunization, the levels of specific antibodies induced by rLaSota-GP3-GP5, rLaSota-GP5, and the commercial vaccine increased gradually and decreased slightly, peaking at 42 dpi (Fig. 3B). Moreover, the levels of above three groups at 42 dpi were significantly

higher than those induced by the rLaSota and PBS control groups ($P < 0.05$). In particular, the level of specific antibodies induced by rLaSota-GP3-GP5 (maximal value of 0.548) was higher than that induced by rLaSota-GP5 and the commercial vaccine with no significant difference, which indicates that the former was better able to stimulate the production of specific antibodies.

At 14 dpi, rLaSota-GP3-GP5, rLaSota-GP5, and commercial vaccine all induced neutralizing antibodies against the HP-PRRSV and there was an upward trend in the levels of neutralizing antibody titers (Fig. 3C). The neutralizing antibody titers of the above three groups at 42 dpi reached 25.49 ± 2.65, 12.19 ± 1.51, and 16.93 ± 2.89,
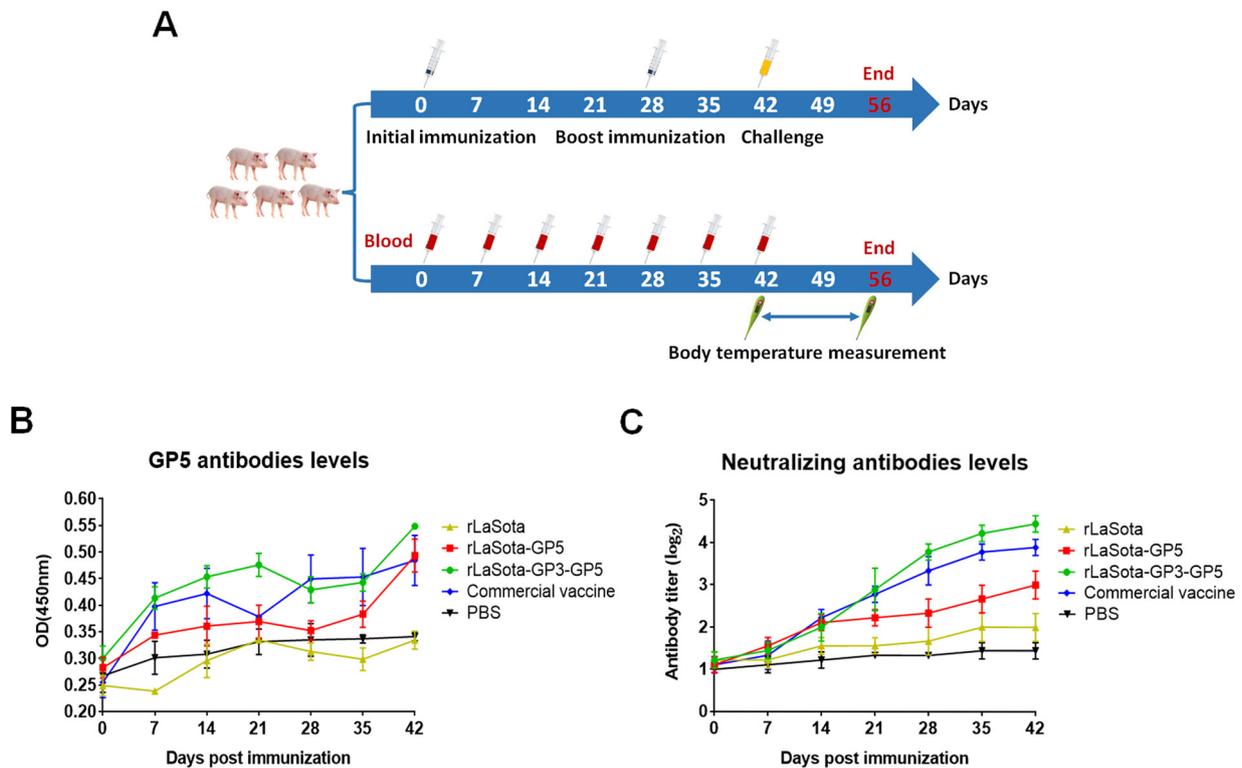
Fig. 3. Changes of antibody levels after immunization. Immunization and challenge programs for the entire experimental cycle were included in a schematic diagram (A). Serum samples of pigs were collected and separated at 0, 7, 14, 21, 28, 35 and 42 dpi. The levels of GP5-specific antibodies in the serum samples of each group were determined by ELISA (B). The neutralizing antibody levels in the serum samples were determined as described in the materials and methods (C). Data represent the mean ± SD of each group.

respectively. The neutralizing antibody titer of the rLaSota-GP3-GP5 group was 1.51 times higher than that of the commercial vaccine group ($P < 0.05$). Collectively, these results demonstrate that the recombinant virus, rLaSota-GP3-GP5, induced a potent antibody response in pigs.

3.4. Recombinant virus rLaSota-GP3-GP5 enhanced cytokine secretion in immunized pigs

Since cytokine production is an important indicator for evaluation of the immune response, serum samples were collected and separated at 14 dpi and 42 dpi to determine the levels of IL-4 and IFN-γ in pigs by ELISA. Overall, the cytokine levels at 42 dpi were generally higher than those at 14 dpi (Fig. 4A and B). At 42 dpi, the concentration of IL-4 and IFN-γ induced in the rLaSota group was 1.64 and 2.25 times higher than that in the rLaSota-GP5 group, respectively ($P < 0.05$) (Fig. 4A). This indicates that the insertion of the exogenous gene may impact the ability of the virus to induce cytokine production in response to NDV. Interestingly, the rLaSota-GP3-GP5 group induced superior cytokine levels at 42 dpi (Fig. 4B). In particular, the level of IFN-γ produced by the rLaSota-GP3-GP5 group (maximal value of 999.42 pg/mL) was 1.34 times higher than that of the rLaSota group ($P < 0.05$), suggesting that the expression of the GP3 protein may promote interferon secretion. In addition, there was a significant difference in the level of IFN-γ between the rLaSota-GP3-GP5 and commercial vaccine groups (450.49 pg/mL) ($P < 0.01$). These results demonstrate that rLaSota-GP3-GP5 markedly enhanced the levels of cytokine secretion.

3.5. Recombinant virus rLaSota-GP3-GP5 stimulated lymphocyte proliferation

To further evaluate the cellular immune responses induced by rLaSota-GP3-GP5, a lymphocyte proliferation assay was performed with

CCK-8. The levels of proliferation in the PRRSV-stimulated groups were higher than those of the Con A-stimulated groups (Fig. 4C). Following stimulation with HP-PRRSV, the SI value of the rLaSota-GP3-GP5 group (maximal value of 2.90) was 1.30 times higher than that of the rLaSota-GP5 group ($P < 0.05$). However, there was no significant difference in the SI values between the commercial vaccine group and the rLaSota-GP3-GP5 group. These results demonstrate that rLaSota-GP3-GP5 could stimulate a dominant lymphocyte proliferation response.

3.6. Recombinant virus rLaSota-GP3-GP5 protected immunized pigs against viral challenge

To evaluate the protective efficacy of the recombinant vaccines, the pathological responses were evaluated in each of the immunized groups of pigs. None of the pigs died from viral infection within two weeks of challenge with the HP-PRRSV GD strain. The mean body temperature of the PBS group increased as much as to 41.5℃. In contrast, the body temperature did not significantly fluctuate in any of the other groups. There was a salient period during which the mean temperature increased in all groups from 3 dpc to 7 dpc. The mean temperature then gradually dropped to nearly 40℃ (Fig. 5A). The changes in the mean temperature of the rLaSota-GP3-GP5 group were more stable than those of the PBS group.

At 14 days post-challenge, the pigs in the PBS group showed diffuse consolidation in the lungs, inguinal and submandibular lymph node enlargement upon pathological examination. Histological examination revealed severe structure destruction of the alveolar and thickening of alveolar walls. No obvious lesions were found in immunized pigs (pictures not showed). Importantly, blood PRRSV viral load was commonly used to monitor the vaccines efficacy. The viral load in blood of the rLaSota-GP3-GP5 group was lower than in the other four groups. Especially, it was significantly lower than that of the rLaSota-GP5 group (41.69 times, $P < 0.05$) and PBS group (83.18 times, $P < 0.01$).
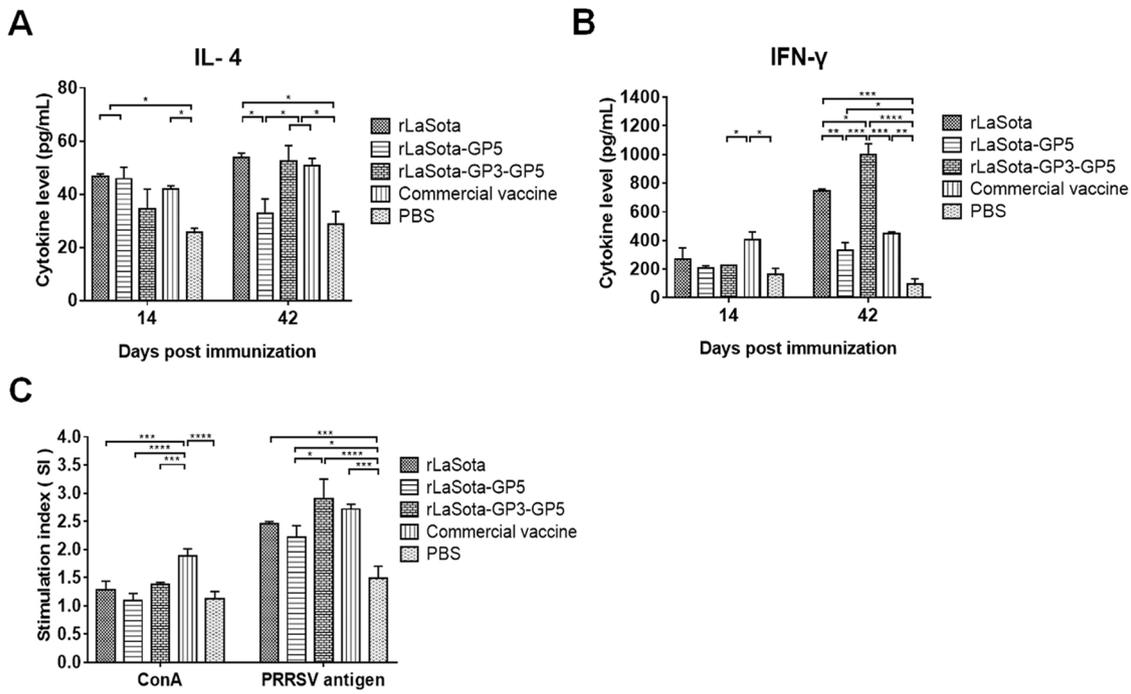
A

IL- 4

B

IFN-γ

C

Fig. 4. Changes of cellular immune levels after immunization. IL-4 (A) and IFN-γ (B) levels in serum samples at 14 dpi and 42 dpi were determined with a commercial ELISA kit according to the manufacturer's instructions, respectively. Lymphocyte proliferation index between the PRRSV antigen and Con A group were determined (C). Data represent the mean ± SD of each group.

Besides, the viral load in the hearts, lungs, and kidneys of the rLaSota-GP3-GP5 and commercial vaccine groups was significantly lower than that in the PBS, rLaSota, and rLaSota-GP5 groups ($P < 0.05$) (Fig. 5B). There was no significant difference in the viral loads in the organs between the rLaSota-GP3-GP5 and commercial vaccine groups, except for the hearts, in which the value of commercial vaccine was 22.39 times higher than that of the rLaSota-GP3-GP5 group ($P < 0.05$). These results demonstrate that the pigs immunized with rLaSota-GP3-GP5 had sufficient protective advantages against HP-PRRSV infection.

4. Discussion

HP-PRRS is classified as the first category of animal diseases in China, which has recently posed a serious threat to the pig industry. In this study, we constructed two recombinant NDV vaccines expressing the HP-PRRSV GP5 / GP3-GP5 proteins using reverse genetics, termed rLaSota-GP5 and rLaSota-GP3-GP5. After 10 successive passages, the recombinant viruses displayed good stability and maintained the same growth kinetics and virulence as rLaSota. In pig immunization experiments, rLaSota-GP3-GP5 induced the production of specific neutralizing
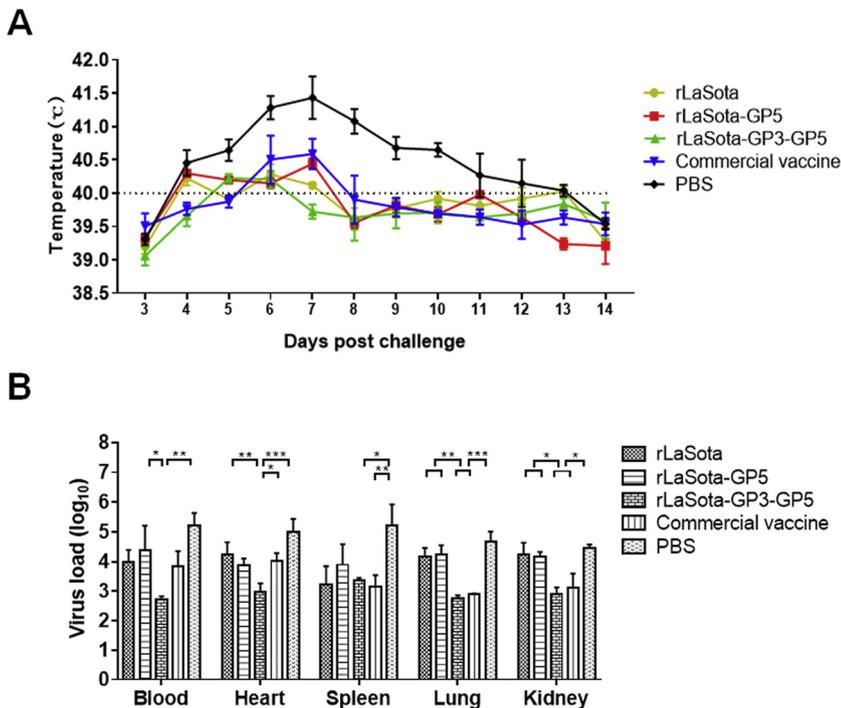
A

B

Fig. 5. Assessment of protective effect of the recombinant candidate vaccines after challenge. The fluctuation in body temperature of pigs in all groups was monitored for 14 days (A). The blood, heart, spleen, lung and kidney samples were collected at 14 dpc. Quantitative real-time PCR was performed to determine the viral load in pig samples (B). Data represent the mean ± SD of each group.

antibodies and the secretion of IFN-γ, and significantly stimulated lymphocyte proliferation. Furthermore, the viral load was substantially lower in the organs of rLaSota-GP3-GP5-immunized pigs following the challenge with the HP-PRRSV GD strain. Our findings indicate that rLaSota-GP3-GP5 may be a promising candidate vaccine for HP-PRRS, displaying good safety and immunogenicity.

The ORF5 region is considered one of the most variable regions among the different PRRSV strains (Yu et al., 2012). As a glycosylated protein, the GP5 protein may alter its glycosylation to influence the immunogenicity as a result of amino acid changes (Liang et al., 2018). The HP-PRRSV GD strain is a representative strain of North American-type PRRSV isolated in Guangdong, China and belongs to the lineage 8/ sub-lineage 8.7 of the PRRSV according to the homology division of ORF5 (Guo et al., 2018). Using a comparative sequence analysis of amino acid sequences, the GP5 protein of the GD strain has a high degree of homology with other representative HP-PRRSV strains (HuN4 and TJ strain: 100%; JXA1 strain: 99.5%; and NADC30 strain: 86.5%) and North American-type PRRSV strains (CH-1R strain: 92%; VR2332 strain: 88.5%). Therefore, a recombinant NDV vaccine expressing the GP5 protein of the GD strain is likely to provide protection against PRRS caused by other North American-type HP-PRRSV strains; however, this remains to be further verified.

In the present study, we found that both rLaSota-GP3-GP5 and rLaSota-GP5 possessed the characteristics of an attenuated viral strain, but there were substantial differences in the immune response induced by the two recombinant viruses in pigs. In general, the efficacy of the rLaSota-GP3-GP5 candidate vaccine was superior to that of the rLaSota-GP5 candidate vaccine, demonstrating that the GP3 protein is involved in inducing a potent immune response and can enhance the humoral and cellular immune responses of GP5 synergistically (Chen et al., 2014; Ren et al., 2014). Especially in interferons, there was a sharp contrast between the two candidate vaccines, which indicated that GP3 protein may have the potential to enhance the expression of interferons.

Since the recombinant NDV candidate vaccine possessed viral activity of NDV, it makes sense to compare the immunogenicity between rLaSota-GP3-GP5 and PRRS commercialized live vaccine, rather than inactivated vaccine. A representative PRRS commercialized attenuated vaccine that is widely used was selected to be the control in this study. It was found that the rLaSota-GP3-GP5 vaccine was significantly superior to the commercial vaccine in that higher levels of neutralizing antibodies and interferons were induced. Furthermore, the viral load in the heart and blood in the groups immunized with the commercial vaccine was higher than that of the rLaSota-GP3-GP5-immunized group. Moreover, the fluctuation in body temperature was more obvious in the group immunized with the commercial vaccine compared to those that received rLaSota-GP3-GP5, which indicated that the latter was associated with greater protective efficacy.

The recombinant viruses rescued in this study were derived from chicken embryo allantoic fluid, which is associated with transient infection in pigs. The EID_{50} is the most direct indicator of virulence of NDV and is a widely used to measure vaccine efficacy in animal immunization experiments (Ge et al., 2007). Based on the dose standards described in previous studies and the weight of the experimental pigs, $2 \times 10^{8.0}$ EID_{50}/mL was adjusted as a uniform immunization dose for the pigs in this study. A dose of $1 \times 10^{5.0}$ $TCID_{50}$/mL is the pathogen content introduced in accordance with commercial vaccine specifications. According to these instructions, we conducted immunization experiments with a volume of 2 mL per pig. Despite the different indicators of viral infectivity between rLaSota-GP3-GP5 and commercial vaccine, the results were of comparative value and significance.

Prior to this animal experiment, we conducted a preliminary experiment on immunized pigs. The only difference is that the immunization interval was three weeks as mentioned in other studies (Han et al., 2018). The general trend of antibody production following antigen stimulation was as follows: there was an initial increase followed by a temporary decrease before secondary stimulation, after

which it significantly increased. Unexpectedly, the specific antibody levels in the immunized pigs did not begin to decline at 21 dpi but significantly decreased one week after the boost immunization in the preliminary experiment. More substantial immune responses could not be observed. Therefore, we adjusted the immunization interval to four weeks and found that the experimental results were improved compared to those in the preliminary experiment. However, this four-week immunization interval is not necessarily optimal and requires further validation.

The HP-PRRS vaccines currently available on the market are relatively limited and consist primarily of modified live vaccines (Guo et al., 2018). These attenuated vaccines may complicate prevention and control due to the potential of increased virulence (Liu et al., 2018). Recombinant virus vector expressing PRRSV viral proteins was considered one of the important means to improve the immune effect (Hu and Zhang, 2014). Qiu et al. proved that a recombinant pseudorabies virus expressing GP5 protein could protect piglets from clinical signs and reduce pathogenic lesions of the disease in spite of no detectable anti-PRRSV antibodies before challenge (Qiu et al., 2005). Shen et al. constructed recombinant fowlpox virus co-expressing GP5 and GP3 and the results of the pig immunization experiment showed that it conferred partial protection against PRRSV challenge, but no comparison was made with the efficacy of commercial vaccines (Shen et al., 2007). Zhou et al. found that recombinant canine adenovirus 2 expressing GP5 could elicits virus-specific neutralization antibodies and lymphocyte proliferation responses, which were lower than the immune responses of commercial killed vaccines (Zhou et al., 2010). It has been reported that NDV displays excellent immune performance as a vaccine vector (Khattar et al., 2010; Kim and Samal, 2016). In addition to the specific advantages of expressing exogenous genes, the candidate vaccine constructed using this vector may also have the general advantage of producing a large number of interferons which was closely related to the type of gene fragments inserted. In this paper, we reported for the first time that NDV was used as a vaccine vector to express HP-PRRSV GP3 and GP5, and proved that rLaSota-GP3-GP5 could produce partial protection superior to commercial vaccines.

5. Conclusion

This study evaluated the immunogenicity of two recombinant NDV candidate vaccines expressing PRRSV GP3-GP5/GP5 proteins in pigs. Recombinant viruses possessed stable genetic characteristics and attenuated virulence consistent with parental virus. The safety of recombinant viruses in the immunized pigs was confirmed. The insertion of different exogenous fragments would seriously affect the ability of NDV vector to induce interferon. rLaSota-GP3-GP5 was screened as a potential candidate vaccine for the prevention and control of HP-PRRS because of the advantages in specific neutralizing antibodies, cytokines and protection against HP-PRRSV. Our findings will contribute to further research into NDV-vectored PRRS vaccines.

Author contributions

NJ, HL, MT, ZB, and JG designed the experiments. HZ and FN performed the virus rescue and identification experiments. HZ, ZL, GZ, CX, ZH, JZ, PX, XZ, and WW performed pig immunization and the challenge protection experiment. HZ, FN, and ZH analyzed the data. HZ wrote the manuscript. HZ, PX, HL, and NJ reviewed the manuscript.

Declaration of Competing Interest

The authors declared no conflict of interest.

Acknowledgments

Project of China [grant number 2018YFD0500104 and 2018YFD0500803].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108490.

References

An, T.Q., Tian, Z.J., Leng, C.L., Peng, J.M., Tong, G.Z., 2011. Highly pathogenic porcine reproductive and respiratory syndrome virus. Asia. Emerg. Infect. Dis. 17, 1782–1784. https://doi.org/10.3201/eid1709.110411.

Brito, B., Dee, S., Wayne, S., Alvarez, J., Perez, A., 2014. Genetic diversity of PRRS virus collected from air samples in four different regions of concentrated swine production during a high incidence season. Viruses-Basel 6, 4424–4436. https://doi.org/10.3390/v6114424.

Bu, X.F., Li, M., Zhao, Y.H., Liu, S., Wang, M.B., Ge, J.Y., Bu, Z.G., Yan, Y.L., 2016. Genetically engineered Newcastle disease virus expressing human interferon-1 induces apoptosis in gastric adenocarcinoma cells and modulates the Th1/Th2 immune response. Oncol. Rep. 36, 1393–1402. https://doi.org/10.3892/or.2016.4925.

Bukreyev, A., Huang, Z., Yang, L., Elankumaran, S., St Claire, M., Murphy, B.R., Samal, S.K., Collins, P.L., 2005. Recombinant newcastle disease virus expressing a foreign viral antigen is attenuated and highly immunogenic in primates. J. Virol. 79, 13275–13284. https://doi.org/10.1128/JVI.79.21.13275-13284.2005.

Chen, J.Z., Wang, Q., Bai, Y., Wang, B., Zhao, H.Y., Peng, J.M., An, T.Q., Tian, Z.J., Tong, G.Z., 2014. Identification of two dominant linear epitopes on the GP3 protein of highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV). Res. Vet. Sci. 97, 238–243. https://doi.org/10.1016/j.rvsc.2014.07.011.

Chen, N.H., Trible, B.R., Kerrigan, M.A., Tian, K.G., Rowland, R.R.R., 2016. ORF5 of porcine reproductive and respiratory syndrome virus (PRRSV) is a target of diversifying selection as infection progresses from acute infection to virus rebound. Infect. Genet. Evol. 40, 167–175. https://doi.org/10.1016/j.meegid.2016.03.002.

DiNapoli, J.M., Yang, L.J., Samal, S.K., Murphy, B.R., Collins, P.L., Bukreyev, A., 2010. Respiratory tract immunization of non-human primates with a Newcastle disease virus-vectored vaccine candidate against Ebola virus elicits a neutralizing antibody response. Vaccine 29, 17–25. https://doi.org/10.1016/j.vaccine.2010.10.024.

Feng, Y., Zhao, T., Nguyen, T., Inui, K., Ma, Y., Nguyen, T.H., Nguyen, V.C., Liu, D., Bui, Q.A., To, L.T., Wang, C., Tian, K., Gao, G.F., 2008. Porcine respiratory and reproductive syndrome virus variants, Vietnam and China, 2007. Emerg. Infect. Dis. 14, 1774–1776. https://doi.org/10.3201/eid1411.071676.

Ge, J.Y., Deng, G.H., Wen, Z.Y., Tian, G.B., Wang, Y., Shi, J.Z., Wang, X.J., Li, Y.B., Hu, S., Jiang, Y.P., Yang, C.L., Yu, K.Z., Bu, Z.G., Chen, H.L., 2007. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. J. Virol. 81, 150–158. https://doi.org/10.1128/JVI.01514-06.

Gonin, P., Mardassi, H., Gagnon, C.A., Massie, B., Dea, S., 1998. A nonstructural and antigenic glycoprotein is encoded by ORF3 of the IAF-Klop strain of porcine reproductive and respiratory syndrome virus. Arch. Virol. 143, 1927–1940. https://doi.org/10.1007/s007050050430.

Guo, Z.H., Chen, X.X., Li, R., Qiao, S.L., Zhang, G.P., 2018. The prevalent status and genetic diversity of porcine reproductive and respiratory syndrome virus in China: a molecular epidemiological perspective. Virol. J. 15. https://doi.org/10.1186/s12985-017-0910-6.

Han, J., Ma, H., Cao, L., Jing, J., Xiao, P., Sun, W., Xie, C., Wen, S., Li, Y., Tian, M., Lu, H., Jin, N., 2018. Immunogenicity of recombinant vaccinia virus vaccines co-expressing GP3/GP5 of European PRRSV and Cap protein of PCV2 in pigs. Appl. Microbiol. Biotechnol. 102, 1145–1154. https://doi.org/10.1007/s00253-017-8661-2.

Hu, J., Zhang, C., 2014. Porcine reproductive and respiratory syndrome virus vaccines: current status and strategies to a universal vaccine. Transbound. Emerg. Dis. 61, 109–120. https://doi.org/10.1111/tbed.12016.

Huang, Y., Li, Z., Li, J., Yibo, K., Yang, L., Mah, C.K., Liu, G., Yu, B., Wang, K., 2019. Efficacy evaluation of three modified-live PRRS vaccines against a local strain of highly pathogenic porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 229, 117–123. https://doi.org/10.1016/j.vetmic.2018.12.016.

Katz, J.B., Shafer, A.L., Eernisse, K.A., Landgraf, J.G., Nelson, E.A., 1995. Antigenic differences between European and American isolates of Porcine Reproductive and Respiratory Syndrome Virus (Prrsv) are encoded by the carboxyterminal portion of viral open reading Frame-3A. Vet. Microbiol. 44, 65–76. https://doi.org/10.1016/0378-1135(94)00113-B.

Khattar, S.K., Collins, P.L., Samal, S.K., 2010. Immunization of cattle with recombinant Newcastle disease virus expressing bovine herpesvirus-1 (BHV-1) glycoprotein D induces mucosal and serum antibody responses and provides partial protection against BHV-1. Vaccine 28, 3159–3170. https://doi.org/10.1016/j.vaccine.2010.02.051.

Kim, S.H., Samal, S.K., 2016. Newcastle disease virus as a vaccine vector for development of human and veterinary vaccines. Viruses-Basel 8. https://doi.org/10.3390/v8070183.

Kimpston-Burkgren, K., Correas, I., Osorio, F.A., Steffen, D., Pattnaik, A.K., Fang, Y., Vu, H.L.X., 2017. Relative contribution of porcine reproductive and respiratory syndrome virus open reading frames 2-4 to the induction of protective immunity. Vaccine 35, 4408–4413. https://doi.org/10.1016/j.vaccine.2017.06.061.

Kortekaas, J., de Boer, S.M., Kant, J., Vloet, R.P.M., Antonis, A.F.G., Moormann, R.J.M., 2010. Rift Valley fever virus immunity provided by a paramyxovirus vaccine vector. Vaccine 28, 4394–4401. https://doi.org/10.1016/j.vaccine.2010.04.048.

Liang, W., Zhao, T., Peng, Z., Sun, Y., Stratton, C.W., Zhou, D., Tang, X., Tian, Y., Chen, H., Wu, B., 2018. Epidemiological and genetic characteristics of porcine reproductive and respiratory syndrome virus circulating in central and South China in 2016. Acta Trop. 190, 83–91. https://doi.org/10.1016/j.actatropica.2018.11.004.

Liu, C., Zhang, W., Gong, W., Zhang, D., She, R., Xu, B., Ning, Y., 2015. Comparative respiratory pathogenicity and dynamic tissue distribution of chinese highly pathogenic porcine reproductive and respiratory syndrome virus and its attenuated strain in piglets. J. Comp. Pathol. 153, 38–49. https://doi.org/10.1016/j.jcpa.2015.04.002.

Liu, P., Bai, Y., Jiang, X., Zhou, L., Yuan, S., Yao, H., Yang, H., Sun, Z., 2018. High reversion potential of a cell-adapted vaccine candidate against highly pathogenic porcine reproductive and respiratory syndrome. Vet. Microbiol. 227, 133–142. https://doi.org/10.1016/j.vetmic.2018.10.004.

Nakaya, T., Cros, J., Park, M.S., Nakaya, Y., Zheng, H.Y., Sagrera, A., Villar, E., Garcia-Sastre, A., Palese, P., 2001. Recombinant Newcastle disease virus as a vaccine vector. J. Virol. 75, 11868–11873. https://doi.org/10.1128/JVI.75.23.11868-11873.2001.

Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76, 4241–4250. https://doi.org/10.1128/JVI.76.9.4241-4250.2002.

Qiu, H.J., Tian, Z.J., Tong, G.Z., Zhou, Y.J., Ni, J.Q., Luo, Y.Z., Cai, X.H., 2005. Protective immunity induced by a recombinant pseudorabies virus expressing the GP5 of porcine reproductive and respiratory syndrome virus in piglets. Vet. Immunol. Immunopathol. 106, 309–319. https://doi.org/10.1016/j.vetimm.2005.03.008.

Ren, J., Lu, H., Wen, S., Sun, W., Yan, F., Chen, X., Jing, J., Liu, H., Liu, C., Xue, F., Xiao, P., Xin, S., Jin, N., 2014. Enhanced immune responses in pigs by DNA vaccine co-expressing GP3 and GP5 of European type porcine reproductive and respiratory syndrome virus. J. Virol. Methods 206, 27–37. https://doi.org/10.1016/j.jviromet.2014.05.021.

Shen, G., Jin, N., Ma, M., Jin, K., Zheng, M., Zhuang, T., Lu, H., Zhu, G., Jin, H., Jin, M., Huo, X., Qin, X., Yin, R., Li, C., Li, H., Li, Y., Han, Z., Chen, Y., Jin, M., 2007. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine 25, 4193–4202. https://doi.org/10.1016/j.vaccine.2007.03.010.

Shen, Y., Pan, X., Zheng, X., 2006. Isolation and identification of newcastle disease virus from pig. J. Anhui Agric. Sci. 19, 4941 (In Chinese).

Snijder, E.J., Kikkert, M., Fang, Y., 2013. Arterivirus molecular biology and pathogenesis. J. Gen. Virol. 94, 2141–2163. https://doi.org/10.1099/vir.0.056341-0.

Takikawa, N., Kobayashi, S., Ide, S., Yamane, Y., Tanaka, Y., Higashihara, M., Yamagishi, H., 1997. Early serodiagnosis of porcine reproductive and respiratory syndrome virus infection of pigs by detection of slow-reacting and complement-requiring neutralizing antibody. J. Vet. Med. Sci. 59, 31–34. https://doi.org/10.1292/jvms.59.31.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS One 2, e526. https://doi.org/10.1371/journal.pone.0000526.

Wang, X.L., Li, J.X., Jiang, P., Li, Y.F., Zashan, B., Cao, J., Wang, X.W., 2009. GM-CSF fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus increased the immune responses and protective efficacy against virulent PRRSV challenge. Virus Res. 143, 24–32. https://doi.org/10.1016/j.virusres.2009.02.017.

Yu, X.L., Chen, N.H., Wang, L.L., Wu, J.J., Zhou, Z., Ni, J.Q., Li, X.D., Zhai, X.Y., Shi, J.S., Tian, K.G., 2012. New genomic characteristics of highly pathogenic porcine reproductive and respiratory syndrome viruses do not lead to significant changes in pathogenicity. Vet. Microbiol. 158, 291–299. https://doi.org/10.1016/j.vetmic.2012.02.036.

Zhou, J.X., Xue, J.D., Yu, T., Zhang, J.B., Liu, Y., Jiang, N., Li, Y.L., Hu, R.L., 2010. Immune responses in pigs induced by recombinant canine adenovirus 2 expressing the glycoprotein 5 of porcine reproductive and respiratory syndrome virus. Vet. Res. Commun. 34, 371–380. https://doi.org/10.1007/s11259-010-9364-7.

Zhou, L., Yang, H.C., 2010. Porcine reproductive and respiratory syndrome in China. Virus Res. 154, 31–37. https://doi.org/10.1016/j.virusres.2010.07.016.