Construction and immunological evaluation of recombinant adenovirus vaccines co-expressing GP3 and GP5 of EU-type porcine reproductive and respiratory syndrome virus in pigs

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ABSTRACT. Porcine reproductive and respiratory syndrome virus (PRRSV) keeps causing economic damages in the swine sector across the globe. There has been emergence of the European (EU) genotype of porcine reproductive and respiratory syndrome virus (Genotype-I PRRSV) in China in recent years. The presently available vaccines cannot unable to provide safeguard against PRRSV infection completely. This study was aimed to construct recombinant adenovirus expressing the ORF3 and ORF5 genes of the EU-type PRRSV strain. Then, the recombinant adenovirus vaccines for EU-type PRRSV (rAd-E3518, rAd-E35, rAd-E3 and rAd-E5) which we constructed and evaluated were constructed and identified by western blot and PCR. All recombinant adenovirus vaccines were evaluated for humoral and cellular responses and EU-type PRRSV challenge in pigs. The results showed that the group of rAd-E3518+Quil A developed higher GP3 and GP5 specific antibody responses compared to the group of rAd-E3518. The majority of the neutralizing antibody titers were higher than 1:16 (P<0.05), the fusion of IL-18 has increased significantly PRRSV-stimulated secretion of IFN-γ and IL-4 in porcine serum, the group of rAd-E3518+Quil A produced highest T-lymphocytes (CD3+CD4+ and CD3+CD8+ T cells) proliferative in peripheral blood of pigs. The animals were challenged with the EU-type PRRSV strain and the viral load was detected in the several tissues, the viral load of rAd-E3518 and rAd-E3518+Quil A were lower than the wild-type adenovirus group. Our findings provide evidence to confirm that the recombinant adenovirus vaccine can protect pigs from EU-PRRSV infection.

KEY WORDS: EU-type porcine reproductive and respiratory syndrome virus (PRRSV), the recombinant adenovirus, vaccine

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Porcine reproductive and respiratory syndrome (PRRS) is counted among the most economically significant pig illness across the globe [21], it is single-stranded positive-sense RNA virus and associated with the family Arteriviridae as well as the genus Arteriviridae [4], and followed by spreading all over the world. The genome size of PRRSV approximately amounts to be 15 Kb with a minimum of ten open reading frames (ORFs), coupled with ORF2 through ORF7 code 8 structural proteins, include GP2, E, GP3, GP4, GP5a, GP5, M and N [2, 7, 9, 20]. Out of the same, ORF5 (GP5) is typically put to use for the phylogenetic analyzes owing to its elevated variability. There are two genotypes of PRRSV are available, which is PRRSV- I (European type), and PRRSV- II (American type) [11, 12], both of the kinds have nearly sixty percent sequence homology [15]; American wild-type PRRSV has been sequestered in Europe, whereas Genotype-I PRRSV has been indicated in Asia and North America [18]. In Korea, North American PRRSV and EU-type PRRSV refer to the first batch of PRRSV isolated in 1997 and 2007 [10, 14]. Respectively, Genotype-II PRRSV was mainly reported and presented a significant challenge to prevent and control of PRRSV in

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China. At present, there is no vaccine corresponding to domestic Genotype-I PRRSV. Then, we have constructed a recombinant adenovirus vaccine, which can better stimulate the body’s immunogenicity.

Adenoviral vectors have been demonstrated favorable application and development prospects in the development of vaccines and gene therapy. In addition, human adenovirus type-5 vector (HAdV-5) has been developed and utilized as cancer gene therapy [8]. Interleukin-18 (IL-18) is a unique cytokine which can enhance innate immunity, as well as enhance Th1 and Th2-driven immune responses [13]. Quillaj A (Quil A) has been used in therapeutic drugs and a variety of preventive vaccines, and the cell mediated immune system as well as enhancing antibody production by Quil A [1, 16].

In this study, Four recombinant adenovirus vaccines (rAd-E3518, rAd-E35, rAd-E3 and rAd-E5) were constructed based on the GP3 and GP5 of European LV strain (M96262), Quil A was used as an adjuvant to immunize pigs with individual recombinant adenovirus vaccines and the animal experiments was evaluated their immunogenicity.

MATERIALS AND METHODS

Viruses and cells
HEK-293 and MARC-145 cells were grown in the DMEM (10% fetal bovine serum (FBS) and 1% streptomycin). The EU-type PRRSV strain LV was provided by the Dalian Entry-Exit Inspection, Quarantine Bureau. The virus titer was $1 \times 10^4$ TCID$_{50}$/ml.

Construction of plasmids to amplify ORF3-ORF5-IL-18
To amplify the ORF3 and ORF5 genes of PRRSV and porcine IL-18 (synthesized by Shanghai Generay Biotech Co., Ltd.), seven pairs of PCR primers were designed (Table 1). The PCR reaction of ORF3-ORF5-IL-18 and Linker-IL-18 were performed and followed PCR program, which have the same reaction to amplify the gene of ORF3-ORF5-IL-18. All the primers used in this study are shown in Table 1. Four recombinant adenovirus (rAd-E3518, rAd-E35, rAd-E3 and rAd-E5) were constructed and a sequence encoding G4S flexible linker was inserted between the ORF3, ORF5 and IL18 genes in the adenovirus expression plasmid rAd-E35 (Fig. 1).

Western blot
Separation of the cells lysates were carried out by 10% SDS-PAGE, and followed by transferring to the nitrocellulose membrane (Beyotime, Shanghai, China). In addition, the adenovirus vaccines uninfected HEK293 cells were putted as the negative control. The membrane was put overnight in a blocking solution (10 percent fat-free milk) and incubated for 2 hr at room temperature with PRRSV specific antiserum (GP3 and GP5 against PRRSV, preserved in our laboratory), and incubated for 1 hr with rabbits anti-pig IgG, which was conjugated with Horseradish peroxidase (Boshide, Wuhan, China) at the dilution amounting to 1/2,000 in PBST. Western blotting (Beyotime) was used for detection.

Vaccination of pigs with recombinant adenovirus vaccine and challenged by EU-type PRRSV
Piglets aged four to six weeks (five piglets in each group) were immunized with recombinant adenovirus vaccines (rAd-E3, rAd-E5, rAd-E35, rAd-E3518 and rAd-E3518+Quil A) dose of $1.0 \times 10^9$TCID$_{50}$/2 ml, PBS and wild-type adenovirus respectively, and blood samples were collected weekly. Each group was vaccinated and accelerators were injected 21 days later. All groups were challenged with EU-type PRRSV (LV) to assess the level of protection by calculating the viral load at 35 dpi. The experimental design is shown in Fig. 2. Procedures involving animals and their care were conducted in conformity with NIH guidelines (NIH

| Primer Name | Sequence (5′→3′) |
|-------------|------------------|
| ORF3-F      | CCGATCCATGGCTCATCAGTG |
| ORF3-R      | GCTCTAGATATCGTGATGTACTGGG |
| ORF5-F      | GGATCCATGAGATGTTCTCAC |
| ORF5-R      | GCTCTAGAATGTACTTTGGCAAGCTTG |
| ORF5-R (W)  | CTCTAGAGGCGGCTCCAATGCT |
| O3-L-O5-F   | GAGGAGGCTCGAGGAGGGTCTATGAGAATGTGTCTAG |
| O3-L-O5-R   | GCGGCCGATGAGAATGAGGTACAAGTCTAG |

Primers ORF3-F, ORF3-R for full-length ORF3; ORF5-F, ORF5-R for full-length ORF5; ORF3-R (W) lacking the promoter; primers IL-18-F, IL-18-R for full-length IL-18; primers IL-18-L-F, IL-18-L-R for the full-length sequence of IL-18 (the underlined part is the G4S flexible linker); primers O3-L-05-F, O3-L-05-R for the full-length sequence of ORF3-linker-ORF5 (the underlined part is the G4S flexible linker).
Specific antibody detection

The specific antibodies were detected by ELISA method. The inactivated standard antigens of GP3 and GP5 subtype were coated overnight in 96-well plates (Laboratory expression purification). The serum samples were diluted 100 times in PBS containing 0.5% (wt/vol) gelatin and 0.15% Tween 20 (ELISA diluent), then applied in duplicate wells for 1.5 hr incubation at 37°C. The plates were washed five times with PBS and then reacted with a 1:1,000 dilution of HRP-labeled goat anti-mouse IgG (Suzhou Jieen Biotechnology) for 1 hr at 37°C. After another five washes with PBS, the substrate was added (10 mg Ortho-Phenylenediamine [OPD]+20 ml 0.015% hydrogen peroxide in phosphate/citrate buffer). After incubation for 15 min at 37°C, which were terminated with 2N H2SO4. Subsequently, the absorbance values were determined at 492 nm using a Sunrise automated plate spectrophotometer.

Neutralizing antibody detection

Sera from each pig in all of the immunization cohorts were heat-inactivated for 0.5 hr at the temperature of 56°C. The 150 TCID50/ml of LV strain was inactivated at 37°C for 1 hr, and then diluted by DMEM containing 2% FBS twice continuously. Subsequently, the mixture was transferred to a single layer of MARC-145 cells and then incubated with 5% CO2 for 4 days at 37°C. Finally, according to Spearman-Karber method, the serum dilution with neutralizing antibody titer was calculated, which could protect 50% of the pore from CPE.
Cytokines secretion assay

Detected serum IL-4, and IFN-γ were followed according to the manufacturer’s instructions (ELISA Ready-SET-Go!, eBioscience, San Diego, CA, U.S.A.).

Analysis of CD4+ and CD8+ T-lymphocytes

At 21, 35 and 45 dpi, the swine peripheral bloods lymph cells were isolated and incubated with anti-CD3+ antibody used for staining, PE mouse anti-pig CD8+ and FITC mouse anti-pig CD4+ were used in pig (BD Biosciences Pharmingen, San Diego, CA, U.S.A.).

Measurements of viremia and the tissue virus loads in pigs

On 14 days subsequent to the challenge, three pigs in each group were euthanized randomly, and their heart, liver, lung, mesenteric lymph nodes and blood samples were collected to detect viral load. In addition, the total RNA was extracted by using the Viral RNA Mini kit (Sangon Biotech, Shanghai, China) according to the manufacturer’s rules. Then, the synthesis of the complementary cDNA carried out with the help of a Reverse Transcription Kit (TaKaRa Biotechnology, Dalian, China) in accordance with the directions of the manufacturer. The reverse transcriptase nested polymerase chain reaction (RT-PCR) was performed for the PRRSV ORF7 gene amplification to establish a quantitative fluorescence detection method (absolute quantification) which detected the PRRSV viral load in blood and tissues. The primers were designed by referring to the sequences of the EU-PRRSV ORF7 gene using the primers 5′-ATGGCCAGCCAGTCAATCA-3′ and 5′-TCGCCCTAATTGAATAGGTG-3′. The reaction was performed at 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec and 53°C for 45 sec.

Statistical analysis

All data were analyzed using the GraphPad Prism software (Version 5.0) and presented as mean ± S.D. The cytokines data were evaluated by one-way repeated measurement ANOVA. Differences were considered significant (P<0.05).

RESULTS

Western blot detection of recombinant proteins GP3 and GP5 proteins expression from EU-type PRRSV

Western Blot showed that the expression protein of rAd-E3 was 30 kDa; the expression of rAd-E5 was 22 kDa and the co-expression of GP3 and GP5 protein in rAd-E35 was 30 kDa and 22 kDa respectively (Fig. 3). These results indicated that the rAd-E35, rAd-E3 and rAd-E5 plasmids expressed well in HEK-293 cells.

Detection of specific antibodies in immune pigs

Next we tested the ability of the recombinant adenoviruses to induce an immune response specific to the PRRSV protein expressed by collecting weekly bloods, the concentration of viral-specific antibodies was detected by indirect ELISA test. The results showed that the antibody level of immunized groups increased significantly from the first week to the second week. In addition, the levels of anti-GP3 antibody of rAd-E3518 and rAd-E3518+Quil A were significant different compared to the group that received the wild-type virus (P<0.01). Only the anti-GP5 antibody level of rAd-E3518 immunized group was higher than compared to the group that received the wild-type virus (P<0.05). After three weeks of immunization, only the level of anti-GP3 antibody of the rAd-E3518 group increased, while the other groups experienced minimal changes; however, the difference was not significant (P>0.05). The level of anti-GP5 antibody increased in rAd-E5 immunization group, while the other groups exhibited minimal changes.

After immunization, the level of anti-GP3 and GP5 antibodies similarly increased, with the highest levels were found in rAd-E3518 and rAd-E3518+Quil A immunized groups at the fourth week, but the difference was not significant (P>0.05). Five weeks after immunization, the anti-GP3 and GP5 antibody in rAd-E3518+Quil A group were higher than those in other groups; in particular, the levels were 1.13 times and 1.15 times higher than the group immunized with rAd-E3518. These results indicate that the use of an adjuvant provides enhanced production of PRRSV-specific antibodies. After immunization, the level of PRRSV-
specific antibodies increased in the blood, indicating that recombinant adenovirus-based vaccines can stimulate the production of humoral immunity; however, this response was not extremely potent (Fig. 4).

**Detection of neutralizing antibodies in sera**

The detect PRRSV neutralizing antibody titers at 0, 7, 14, 21, 28 and 35 dpi, which in pig immunized with the recombinant adenovirus vaccine rAd-E3518+Quil A (1:19.28) was significantly higher than the groups immunized of rAd-E3 and rAd-E5 (P<0.05), and peaked at 35 dpi was 1:11 and 1:12.4 (Table 2). The neutralizing antibody titers of experimental groups were significantly higher than control groups (wild-type adenovirus and PBS) at 35 dpi (P<0.05), the results showed that rAd-E3518+Quil A promoted the neutralizing antibodies well in the body.

**Levels of secreted cytokines IL-4 and IFN-γ after immunization**

At 14 dpi and 35 dpi, the levels of IL-4 and IFN-γ in sera of the experimental groups were higher than negative control groups (wild-type adenovirus and PBS) (P<0.05). At 35 dpi, the levels of IL-4 and IFN-γ in the rAd-E3518+Quil A group were highest, but there was no significant difference in statistics (Fig. 4A and 4B). The results showed that the recombinant adenovirus vaccines could effectively stimulate the body to increase the secretion of Th1 and Th2 cytokines in pigs (Fig. 5).

**Levels of CD4+ and CD8+ T-cell subtype analysis of immunized pigs**

Three weeks post-immunization, the level of CD3+CD4+ and CD3+CD8+ T cells of the experimental groups were higher than negative control groups (wild-type adenovirus and PBS) (P>0.05). After immunization, the level of CD4+ T cells in the rAd-E3 and rAd-E3518 were significantly higher compared to that exhibited by the wild-type group (P>0.05), the rAd-E3518+Quil A group had a highly significant compared with the wild-type control (P<0.01). Moreover, the levels of CD8+ T cells in rAd-E3518 and

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**Table 2.** Anti-porcine reproductive and respiratory syndrome virus (PRRSV) neutralizing antibodies in pigs inoculated with the recombinant adenovirus vaccines

| Immune group          | 7 dpi(b) | 14 dpi     | 21 dpi     | 28 dpi     | 35 dpi     |
|-----------------------|----------|------------|------------|------------|------------|
| rAd-E3                | -c)      | -          | 6.12 ± 1.55| 7.80 ± 0.45| 10.95 ± 1.50|
| rAd-E5                |          | 5.62 ± 1.85| 6.50 ± 0.83| 9.56 ± 1.89| 12.39 ± 0.81|
| rAd-E35               |          | 7.80 ± 2.34| 8.83 ± 0.61| 12.61 ± 1.94| 16.34 ± 0.39|
| rAd-E3518             |          | 5.49 ± 1.82| 6.99 ± 1.56| 12.50 ± 0.94| 16.34 ± 1.99|
| rAd-E3518+Quil A      |          | 8.56 ± 1.00| 9.89 ± 1.66| 13.34 ± 2.11| 19.28 ± 2.94|
| PBS                   |          | -          | -          | -          | -          |
| Wild-type adenovirus  |          | -          | -          | -          | -          |

(a) Serum samples from vaccinated piglets were individually analyzed to determine the neutralizing antibody titers. b) The number of days after primary vaccination (days post challenge). c) Neutralizing antibodies are negative or the titer is less than 1.2.

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![Fig. 4.](image-url)
rAd-E3518+Quil A immunization groups were the highest, which compared with the negative control group. (wild-type adenovirus and PBS) (Fig. 6). The protective ability of PRRSV vaccine was tested after challenge. Compared with wild type and PBS control group, the level of T cells in peripheral blood of vaccinated animals was higher.

**Viremia and tissue-specific viral loads after PRRSV challenge**

After two weeks of PRRSV challenge, heart, liver, lung, mesenteric lymph nodes and blood were taken from which we euthanized pigs in the recombinant adenovirus group and the viral titers were analyzed by quantitative PCR. The results showed that the viral load of main organs in immunized groups were lower than that in the wild-type and PBS controls. The viral load of the mesenteric lymph nodes, spleen, lungs and bloods were lowest in the rAd-E3518 group and rAd-E3518+Quil A group compared to the other immunized groups, as well as wild-type control (P<0.05). In addition, the rAd-E3518+Quil A group’s viral load of the heart and bloods were only 4 or 7% of the negative control group. However, the viral load of the heart, liver and blood in rAd-E3518+Quil A group were 5, 5 and 5% of the wild-type group. Therefore, these results indicated that the recombinant adenovirus vaccine candidates can reduce the viral load of immunized pigs after PRRSV challenge (Fig. 7).
DISCUSSION

The Europe strain of PRRSV (EU-PRRSV) is highly prevalent throughout European countries and has recently been reported in North America and Asia [6]. Moreover, the virus has also been identified in the provinces of Beijing, Shanghai, Jiangsu, Inner Mongolia, Liaoning and other regions in China [5]. Thus, the emergence of EU-PRRSV in China brings new challenges for the prevention and control of the disease. Currently, there is no available vaccine against EU-PRRSV in China, and the prevention of the disease has become an important and growing concern. IL-18 can regulate the antiviral response and other functions of the immune system [22], it also can promote Th1 cytokine secretion and promote T cell proliferation, improve the activity of NK cells, and enhance both specific and nonspecific immune functionality. Our data clearly indicated that immunization with rAd-E3518+Quil A, co-expressing the modified GP3 and GP5 proteins of PRRSV, could confer higher protective efficacy against virulent EU-type PRRSV challenge and induce PRRSV-specific immune responses compared with the negative control. In particular, the vaccine can produce specific antibodies and reduce the incidence of viremia, indicating that the vaccine is a promising candidate vaccine against EU-PRRSV.

As far as we know, the adenovirus vector has great application prospects, and has applied as a vaccine or therapeutic in a variety of diseases [3, 18]. In the present study, the production of antigen-specific and neutralizing antibodies against GP3 and GP5 were significant. In this study, an FMDV-2A (Foot and Mouth disease virus) linker was inserted between GP3 and GP5 of PRRSV, and co-expression of GP3 and GP5 protein was expressed in an adenovirus vector, Western blot results revealed that the expression of the GP3 and GP5 proteins in the adenovirus vector could also form disulfide-linked heterodimers. Therefore, we speculate that the immunogenicity of rAd-E35 was enhanced due to the formation of a GP3/GP5 heterodimer. The recombinant adenoviruses rAd-E3 and rAd-E5 exhibit protein expression at 30 kDa and 22 kDa, respectively and rAd-E35 can also co-express the GP3 and GP5 proteins at the same time. Therefore, the co-expression of the GP3 and GP5 proteins can be used in the design of a new generation of vaccines against PRRSV infection.

As we mentioned above, the recombinant rAd-E35 and rAd-E3518+Quil A were compared to recombinant rAd-E3 and rAd-E5 which induced a significantly higher PRRSV neutralizing antibody response in immunized pigs. Moreover, a fusion protein strategy has been used in a PRRSV vaccine design and exhibited enhanced immune responses [19]. The results of the detection of previous work had been shown that IL-18 increases CD3+, CD4+ and CD8+ T cells in the peripheral bloods of immunized mice [17]. Therefore, we find that the levels of CD4+ and CD8+ T-cell of rAd-E3518+Quil A vaccination has the potential to improve the cellular immune responses in immunized pigs and the expression of cytokines IL-4 and IFN-γ revealed that the co-expression of both GP3 and GP5 with IL-18 can enhance both humoral and cellular immunity against EU-type PRRSV. After five weeks of post-immunization, in order to confirm the immune effect of rAd-E3518+Quil A, the EU-PRRSV was used for the challenge protection experiment. We evaluated the protective effect of vaccines by detecting viremia and viral load in major organs. Then we found that the rAd-E3518+Quil A immunized group exhibited a more robust immune response and provided enhanced protection compared to the vaccinated other vaccinated groups. This experiment will play a certain role in further understanding the immunity and control of EU-PRRSV.

In conclusion, our findings indicate that the Ad-vector recombinant vaccine (rAd-E3518+Quil A) co-expressing the modified GP3 and GP5 proteins of PRRSV can induce EU-PRRSV immune response in the virus’s natural host. Importantly, some protection was also demonstrated following challenge with EU-PRRSV. Although the mechanisms responsible for protection...
following an EU-PRRSV infection remain unclear, it is necessary to further elucidate whether pregnant sows can be protected by this vaccine against reproductive failure.

CONFLICT OF INTEREST. The authors have declared that no competing interests exist.

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