Brazilian Journal of Microbiology (2019) 50:279–286
https://doi.org/10.1007/s42770-018-0022-5

VETERINARY MICROBIOLOGY - RESEARCH PAPER

Evaluation on the efficacy and immunogenicity of recombinant DNA plasmids expressing S gene from porcine epidemic diarrhea virus and VP7 gene from porcine rotavirus

Yue Yin[1] · Ling Zhu[1,2] · Pengjuan Liu[1] · Jun Zhao[1] · Yi Fan[1] · Xiangang Sun[1] · Zhiwen Xu[1,2]

Received: 15 January 2018 / Accepted: 2 October 2018 / Published online: 7 December 2018

Abstract

Porcine rotavirus (PoRV) and porcine epidemic diarrhea virus (PEDV) usually co-infect pigs in modern large-scale piggery, which both can cause severe diarrhea in newborn piglets and lead to significant economic losses to the pig industry. The VP7 protein is the main coat protein of PoRV, and the S protein is the main structural protein of PEDV, which are capable of inducing neutralizing antibodies in vivo. In this study, a DNA vaccine pPI-2.EGFP.VP7.S co-expressing VP7 protein of PoRV and S protein of PEDV was constructed. Six 8-week-old mice were immunized with the recombinant plasmid pPI-2.EGFP.VP7.S. The high humoral immune responses (virus specific antibody) and cellular immune responses (IFN-γ, IL-4, and spleen lymphocyte proliferation) were evaluated. The immune effect through intramuscular injection increased with plasmid dose when compared with subcutaneous injection. The immune-enhancing effect of IFN-α adjuvant was excellent compared with pig spleen transfer factor and IL-12 adjuvant. These results demonstrated that pPI-2.EGFP.VP7.S possess the immunological functions of the VP7 proteins of PoRV and S proteins of PEDV, indicating that pPI-2.EGFP.VP7.S is a candidate vaccine for porcine rotaviral infection (PoR) and porcine epidemic diarrhea (PED).

Keywords PoRV · PEDV · Co-expression · DNA vaccine · Immune effect

Introduction

PoRV and PEDV both can cause severe enteric diseases in newborn piglets which are characterized by extremely high mortality and devastating economic consequences for swine industry [4, 14]. PoRV belong to the *genus Rotavirus* in the *family Reoviridae* [14], which can infect pigs of all ages, especially piglets, and cause diarrhea and vomit dehydration. Since it was first isolated from swine waste in 1974, PoRV has become a global epidemic these days [7]. Even though some genes in PoRV genome can limit the host selection, some animal rotavirus is likely across host barrier to infect humans, seriously affecting the health of the human intestine. A previous study of rotaviral strains circulating among Brazilian children provided the evidence of transmission of RVC from swine to human [4].

PEDV is related to PoRV and bears similarities in the clinical disease and lesions induced, and it is a member of the *Coronaviridae family*, which induces highly contagious acute intestinal diseases, especially in piglets. PEDV was first reported in the European Union in 1978 [16], then quickly spread to other European countries in the 1980s and later to Asian countries, including Japan, Korea, China, and Thailand in 2014 [11]. In 2014, a new PEDV variant (OH851 strain) caused the outbreak of epiduse emic diarrhea in the USA, which led to huge economic losses [19]. This disease approached a mobility of as high as 100% and a mortality of 80–100% in piglets less than 10 days old [15, 16].

To protect animals from PEDV and PoRV infection, vaccination is an effective prevention measure. Although, there are many commercial vaccines, including live attenuated vaccines and inactivated vaccines, like PEDV CV777 strain, which is widely used in Chinese pig farms. PoRV SC201201 strain was isolated by our laboratory and proved to be efficient against PoRV. But it is impossible to be safe and elicit the effective

Responsible Editor: Flávio da Fonseca

✉ Zhiwen Xu
abtcxzw@126.com

[1] College of Veterinary Medicine, Sichuan Agricultural University, Huimin Road 211#, Wenjiang District, Chengdu 611130, Sichuan Province, China

[2] Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Huimin Road 211#, Wenjiang District, Chengdu 611130, Sichuan Province, China

protective responses at the same time. So, these two diseases are still major problems in swine farms [17].

The S protein is the structural surface protein of PEDV which has a high antigenic index, and several studies reported the PEDV S protein plays an important role in binding specific receptor and induction of neutralizing antibodies [8]. The VP7 protein is the outer capsid proteins of PoRV which can induce neutralizing antibodies and has potential to be a good vaccine candidate [18]. Therefore, in this study, we evaluated a combined vaccination approach based on the plasmid-driven expression of two proteins, the PoRV VP7 protein and the PEDV S protein using mice model. This investigation is a first step for developing bivalent vaccines against PoR and PED.

Materials and methods

All animal experiments had been approved by the Laboratory Animal Management Committee of Sichuan Province (Approval Number SYXK (Chuan) 2014-187).

Plasmid, virus, and cell lines

The virulent PoRV SC-R strain [21] and PEDV SC-P strain [25] were isolated and preserved by Veterinary Medicine, Sichuan Agricultural University (Chengdu, China). PEDV CV777 strain and PoRV SC201201 strain were vaccine strains and preserved by Veterinary Medicine, Sichuan Agricultural University (Chengdu, China). pPI-2.EGFP eukaryotic expression vector was constructed and preserved by the Veterinary Medicine, Sichuan Agricultural University (Chengdu, China). IFN-α, IL-12, and pig spleen transfer factor were preserved by Veterinary Medicine, Sichuan Agricultural University (Chengdu, China). BHK-21 cells were grown and maintained in RPMI1640 (Hyclone, USA), supplemented with 10% fetal calf serum (Gibco, USA) at 37 °C with 5% CO_2 in a humidified incubator.

Animal model

Six to 8-week-old mice were purchased from Dashuo (Chengdu, Sichuan). The relevant pathogens (PoRV and PEDV) and antibodies (anti-PoRV and anti-PEDV) were not detected. The mice were fed under controlled conditions with temperature = 18–22 °C and humidity = 40–50%.

Construction of pPI-2.EGFP.VP7.S expression plasmid

PoRV VP7 gene (49–1017 nt) and PEDV S gene (1471–2415 nt) were predicted to be dominant antigen genes using DNA star software. So PoRV VP7 (49-1017 nt) gene was amplified from the total RNA of PoRV SC-R strain by RT-PCR using a sense primer VP7-a (5′-GCGAATTCCATGTATGGTATTGAATATAC-3′) and antisense primer VP7-b (5′-GCGGTACCATAAAAGGCAGCAGAAT-3′), which contain EcoR I (VP7-a) and Kpn I (VP7-b) restriction enzyme sites (underlined). PEDV S gene (1471–2415 nt) was amplified from the total RNA of PEDV SC-P strain by RT-PCR using sense primer S-a (5′-GGTACC*GGTTCTGGATCAGGAGGTTCTGGA*TCAGGAAGTCACGAACAGCCAAT-3′) and antisense primer S-b (5′-GCGGATCCACAATCAACACTAACAGGCGT-3′) which contain Kpn I (S-a) and BamH I (S-b) estriction enzyme sites (underlined) and Linker sequence (italic). All PCR products were purified, digested by restriction enzyme, and ligated into the eukaryotic expression vector pPI-2.EGFP. After transformation, clones were picked and validated by DNA sequencing. The plasmids were designated as pPI-2.EGFP.VP7.S (including PoRV VP7 gene and PEDV S gene).

Expression assay of pPI-2.EGFP.VP7.S in vitro

The BHK-21 cells were transiently transfected with pPI-2.EGFP.VP7.S and empty vector pPI-2.EGFP using Lipefectamine™ 3000 reagent (Invitrogen, USA). Sixty-hour post transfection, the transfected cells were washed with PBS and incubated with RIPA lysis buffer. The expression of the recombinant plasmids was analyzed by western blot with dilute primary antibodies (mouse anti-PoRV and mouse anti-PEDV) and secondary antibodies (HRP-conjugated-sheep-antimouse IgG).

Immunization and sample collection

Eleven test groups of mice (22 per group) were immunized by different approaches two times with different doses with a 2-week interval. The detailed immunization strategies are shown in Table 1. The serum samples were collected at 0, 7, 14, 21, 28, and 42-days post immunization (dpi) and spleen was collected at 0, 14, 28, and 42 dpi.

Enzyme-linked immunosorbent assay (ELISA)

VP7-specific IgG and S-specific IgG in serum of mice were analyzed by an indirect ELISA (Lvshiyuan, Shenzhen, China). Standard sera were added by 100 uL. Serum samples were added by 100 uL/well after 100-fold dilution and then the plate was incubated for 30 min at 37 °C. After washing three times with washing solution, horseradish peroxidase (HRP)-labeled goat anti-mice IgG, the secondary antibody was added, and the plates were incubated for 30 min at 37 °C. After washing, TMB substrate solution were added by 100ul/well and then incubated for 15 min in the dark. The reaction was terminated by the addition of 50 uL stop solution. The optical density (OD) at 450 nm was measured by enzyme-linked immunosorbent assay systems.

Table 1 Groups and immunization in mice

Groups	Numbers	Vaccine	First immunization	Second immunization	Approaches	Adjuvants
A	22	pPI-2.EGFP.VP7.S	100 μg	100 μg	Intramuscular injection	/
B	22	pPI-2.EGFP.VP7.S	200 μg	200 μg	Intramuscular injection	/
C	22	pPI-2.EGFP.VP7.S	50μg	50 μg	intramuscular injection	/
D	22	pPI-2.EGFP.VP7.S	100 μg	100 μg	Subcutaneous injection	/
E	22	pPI-2.EGFP.VP7.S	100 μg	100 μg	Intramuscular injection	2000 IU pig IFN-α
F	22	pPI-2.EGFP.VP7.S	100 μg	100 μg	Intramuscular injection	2000 U pig IL-12
G	22	pPI-2.EGFP.VP7.S	100 μg	100 μg	Intramuscular injection	300-uL pig spleen transfer factor
H	22	pPI-2.EGFP	100 μg	100 μg	Intramuscular injection	
I	22	PoRV SC201201 strain	0.2 mL	0.2 mL	Intramuscular injection	
J	22	PEDV CV777 strain	0.2 mL	0.2 mL	Intramuscular injection	
k	22	PBS	0.2 mL	0.2 mL	Intramuscular injection	

Cytokines secretion assay

IFN-γ in serum of mice were detected by ELISA using commercially available mouse ELISA kits (Endogen, America) following the manufacturer's instructions. Control IFN-γ diluted in PBS was serially-diluted twofold in PBS between 800 and 0 pg/ml and then coated onto ELISA plates overnight at 37 °C; serum of mice were also coated onto ELISA plates and incubated with HRP-conjugated anti-mouse antibodies at 37 °C for 1 h. Substrate solution A and B were added and then incubated for 15 min in the dark. The reaction was terminated by the addition of stop solution. The OD450 values and therefore pg/ml of IFN-γ in immunized mice were determined according to the IFN-γ standard curve.

IL-4 in serum of mice were detected by ELISA using commercially available mouse ELISA kits (Endogen, America) following the manufacturer's instructions. Control IL-4 was serially-diluted twofold in PBS between 160 and 0 pg/ml then coated onto ELISA plates at 37 °C overnight. The ELISA was performed as above, and OD450 values (pg/ml) were determined according to the IL-4 standard curve.

Spleen T lymphocytes proliferation (MTT)

Lymphocyte proliferation was assessed by the MTT method. Mice from each group were sacrificed, and spleens were taken with aseptic manipulation. Single-spleen cell suspensions were prepared; erythrocyte was dissolved with Tris-NH_4Cl. The cell concentration was adjusted to 1×10^7cells/mL with RPMI1640 medium containing 10% fetal bovine serum and then transferred to 96-well plates. One hundred microliters of the medium containing 5 μg/mL concanavalin A was added to each well. The plates were incubated for 72 h at 37 °C with 5% CO_2, subsequently, supplemented with 100ul/well MTT, and then incubated for 3 h additionally. The reactions were terminated by adding 100 ul/well stop solution. Proliferation was determined by OD_{490} values.

Statistical analysis

The experimental data were analyzed by one-way ANOVA using SPSS 16.0 software and the results were plotted using GraphPad Prism5.0 software, and error bars represent standard deviations. The results are expressed as mean ± standard deviation (SD), and $P < 0.05$ and $P < 0.01$ were considered as statistically high and extremely significantly, respectively.

Results

Construction and expression of pPI-2.EGFP.VP7.S in BHK-21 cells

Nine hundred eighty-six and 989 kb DNA fragments were amplified by RT-PCR from PoRV SC-R strain VP7 gene and PEDV SC-P strain S gene. The S gene and VP7 gene fragments were incorporated into pPI-2.EGFP vector. The recombinant plasmid pPI-2.EGFP.VP7.S was characterized by PCR and restriction enzyme analysis (Fig. 1). To verify that recombinant plasmid pPI-2.EGFP.VP7.S was capable of expressing proteins in cells, the expression of a target gene of plasmids in BHK-21 cells was detected by Western blot. As shown in Fig. 2, 98 KD band was observed on the NC membrane (Fig. 2), which is similar to the predicted molecular weight of VP7-S.

The detection of IgG in mice serum

Comparing the immune effects by different immunization approaches

The level of serum antibody against PoRV in group A (intramuscular injection) was significantly higher ($P < 0.01$) than that in group D (subcutaneous injection) at 21, 28, 42 dpi (Fig. 3)a. The serum antibody level against PEDV in group

D was significantly lower compared with group A at 14 dpi ($P < 0.05$), 28 dpi ($P < 0.01$), and 42 dpi ($P < 0.01$) (Fig. 3b).

Comparing the immune effects by different immunization doses

The level of IgG against PoRV in group B at 7, 14, 21 dpi ($P < 0.05$), 28, and 42 dpi ($P < 0.01$) statistically higher than that in group A. During immune period from 7 to 42 days, the level of IgG in group B was significantly higher ($P < 0.01$) than that in group C (Fig. 4a).

PEDV specific antibody in group B was significantly higher ($P < 0.01$) than that in group A at 14, 21, 28, and 42 dpi. The serum antibody level in group C was significantly lower ($P < 0.01$) than that in group A at 14 dpi, and no significant difference was observed ($P > 0.05$) at 28 dpi (Fig. 4b).

Comparing the immune effects by different immunization adjuvant

The serum antibody level against PoRV in group E was significantly higher than that in group A at 7 dpi ($P < 0.05$), and in group F at 28 dpi ($P < 0.05$), at 42 dpi ($P < 0.01$). The serum antibody level in group G was significantly lower ($P < 0.01$) than that in E group at 7–21 dpi, and at 28–42 dpi ($P < 0.05$) (Fig. 5a).

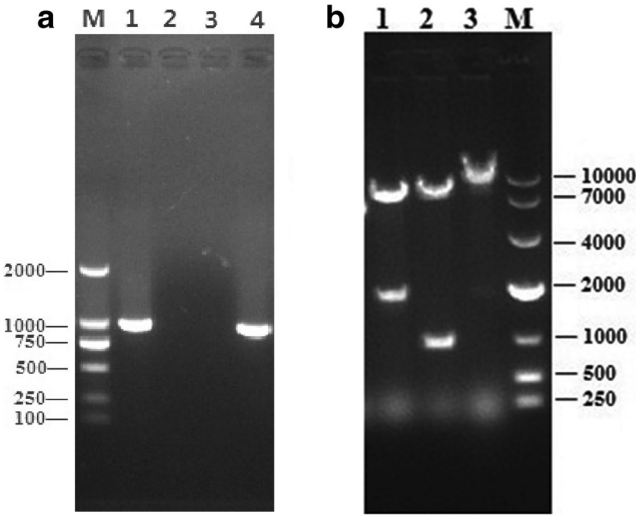

Fig. 1 A Identification of the recombination plasmid pPI-2.EGFP.VP7.S using PCR. Lane M: 2000 bp control size DNA markers lane 1: S gene fragment of PEDV amplified through PCR from pPI-2.EGFP.VP7.S. lanes 2 and 3: Negative control. Lane 4: VP7 gene fragment of PoRV amplified through PCR from pPI-2.EGFP.VP7.S. B Identification of the recombination plasmid pPI-2.EGFP.VP7.S by restriction enzyme digestion. Lane 1: pPI-2.EGFP.VP7.S was digested with EcoR I and BamH I released an insert size 1967 bp and pPI-2.EGFP vector(7611 bp). Lane 2: pPI-2.EGFP.VP7.S was digested with Kpn I and BamH I released an insert size 989 bp and pPI-2.EGFP.VP7 (8600 bp). Lane 3: PPI-2.EGFP.VP7.S undigested vector. Lane M: 10000 bp control size DNA markers

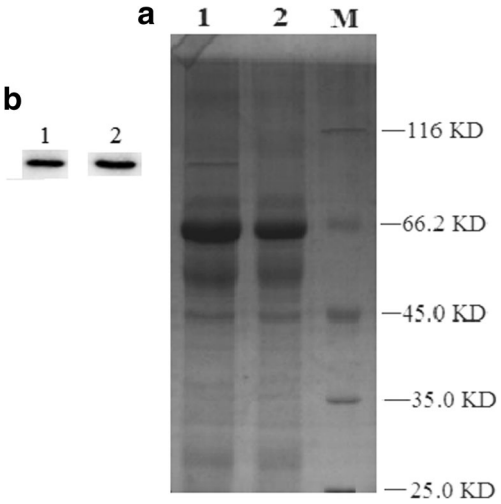

Fig. 2 A: SDS-PAGE detection the expression of pPI-2.EGFP.VP7.S in BHK-21 cells. Lane 1: BHK-21 cells were transfected with pPI-2.EGFP.VP7.S. Lane 2: Negative cells. Lane M: protein marker. B: Western Blot detection the expression of pPI-2.EGFP.VP7.S in BHK-21 cells. Lane 1: Primary antibodies were antiserum of mouse to PEDV. Lane 2: Primary antibodies were antiserum of mouse to PoRV

The serum antibody against PEDV level in group E and group G were significantly higher than that in group F at 14 dpi ($P < 0.01$). The serum antibody level of group E was significantly higher ($P < 0.01$) than that in group G at 28 dpi (Fig. 5b).

Detection of cytokines

The levels of IFN-γ in all immunization groups were significantly higher ($P < 0.01$) than that in group K and group H at 42 dpi. The level of IFN-γ in group B was ($P < 0.01$) higher than that in group A and group C. the level of IFN-γ in group A was higher ($P < 0.01$) than that in group D. There were no significant differences between groups of different immune adjuvants (Table 2a).

The level of IL-4 in group B was significantly higher ($P < 0.01$) than that in group A and group C. Compared with groups of different immunization approaches, the level of IL-4 in group A was significantly higher ($P < 0.05$) than that in group D. Compared with groups of different immunization adjuvants, the level of IL-4 in group E was higher ($P < 0.01$) than that in group F and significantly ($P < 0.05$) higher than that in group G. No significant differences ($P > 0.05$) were observed between group F and group G (Table 2b).

Spleen lymphocyte proliferation

The change of lymphocyte proliferation in spleen was detected by MTT test. The levels of spleen lymphocyte proliferation value increased with a dose-dependent. The spleen lymphocytes proliferation in group B was significantly higher (P< 0.01) than that in group A and group C from 14 dpi.

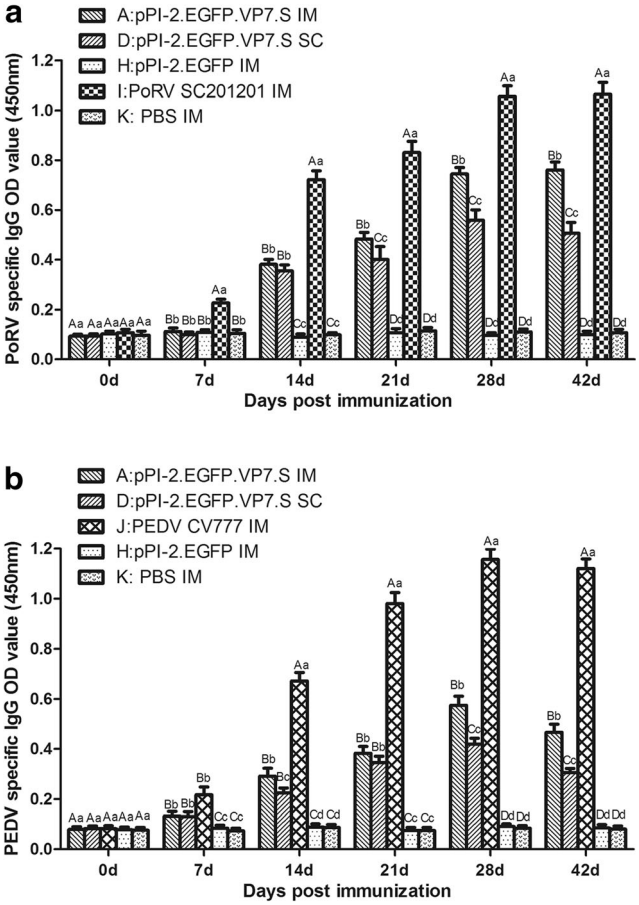

Fig. 3 Humoral immune responses of the mice immunized with 100 μg pPI-2.EGFP.VP7.S intramuscular injection (group A), 100 μg pPI-2.EGFP.VP7.S subcutaneous injection (group D). In addition, the empty vector pPI-2.EGFP (group H), PBS (group K), PoRV SC201201 (group I), and PEDV CV777 (group J) were used as controls. Anti-PoRV serum antibodies (a) and anti-PEDV serum antibodies (b) were detected by ELISA at different time points following the injection of the plasmid DNAs. Different capitals at the same time represent the difference was extremely ($P < 0.01$), different small letters represent the difference was stastically ($P < 0.05$)

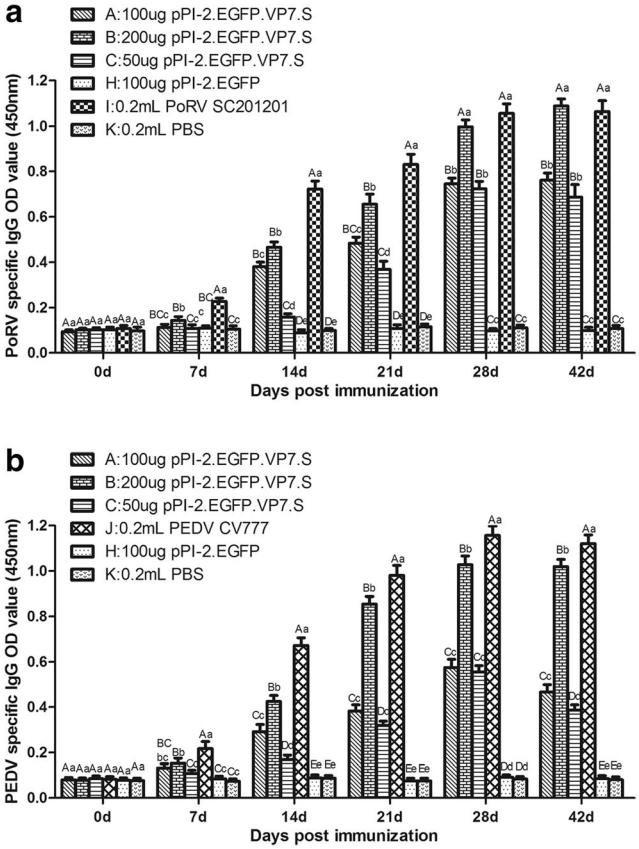

Fig. 4 Humoral immune responses of the mice immunized with 200 μg (group B), 100 μg (group A), 50 μg (group C) pPI-2.EGFP.VP7.S, respectively. In addition, the empty vector pPI-2.EGFP (group H), PBS (group K), PoRV SC201201 (group I), and PEDV CV777(group J) were used as controls. Anti- PoRV serum antibodies (a) and anti-PEDV serum antibodies (b) were detected by ELISA at different time points following the injection of the plasmid DNAs. Different capitals at the same time represent the difference was extremely ($P < 0.01$), different small letters represent the difference was stastically ($P < 0.05$)

The spleen lymphocytes proliferation of group D increased after the primary immunization and reached maximum at 28 dpi, then declined. It was significantly lower ($P < 0.01$) than group A at 28 dpi.

The spleen lymphocyte proliferation in group E was statistically higher ($P < 0.05$) than that in group F and group G at 28 dpi, and was higher ($P < 0.01$) than that in group F and group G at 28–42 dpi. The spleen lymphocyte proliferation in group G was statistically higher ($P < 0.05$) than that in group F at 28–42 dpi (Fig. 6).

Discussion

Vaccination is one of the most effective prevention measures against disease outbreak and epidemic. Compared with traditional vaccines, DNA vaccines are more stable, economical, and easily produced [22]. DNA vaccines against many viral diseases of porcine have been successfully developed and tested, such as DNA vaccines for porcine circovirus 2 [6], polyvalent influenza [9], and transmissible gastroenteritis coronavirus [23]. In this study, we had demonstrated that a DNA vaccine expressed S gene of PEDV, and VP7 gene of PoRV was a potent vaccine that elicited specific serum antibody and non-specific parameters. Besides, neither side effects nor deaths were found in mice post immunization with pPI-2.EGFP.VP7.S.

Choosing exogenous gene is the first and key step to design an efficient vaccine [5]. The S protein is the structural surface potein of PEDV which has a high antigenic index and can induce neutralizing antibodies. The VP7 protein is the outer capsid proteins of PoRV which can induce neutralizing antibodies. Besides, PoRV VP7 gene (49–1017 nt) and PEDV S gene (1471–2415 nt) were predicted to be dominant antigen genes using DNA star software. Therefore, the VP7 gene (49–

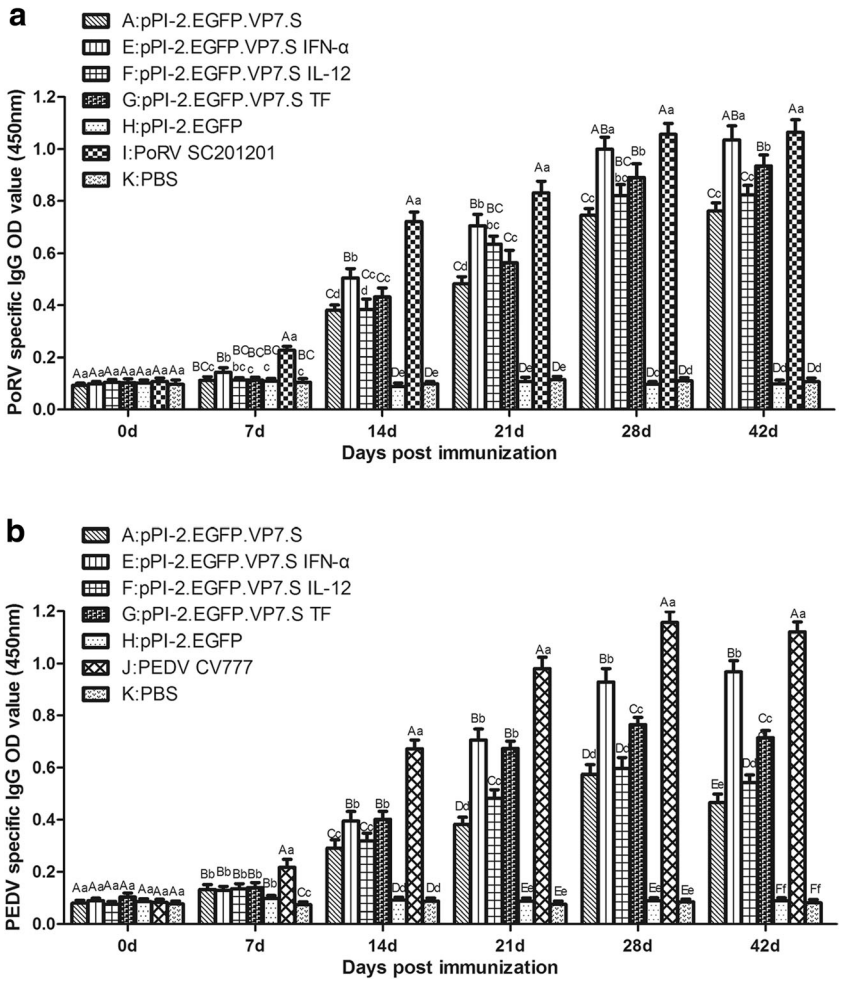

Fig. 5 Humoral immune responses of the mice immunized with 2000 U pig IFN-α (group E), pig IL-12 (group F) and 300 ul pig spleen transfer factor (group G) at 24 h and 48 h after first and secondary immunity. In addition, 100 μg pPI-2.EGFP.VP7.S intramuscular injection (group A), PBS (group K), PoRV SC201201 (group I), and PEDV CV777 (group J) were used as controls Anti- PoRV serum antibodies (a) and anti- PEDV serum antibodies (b) were detected by ELISA at different time points following the injection of the plasmid DNAs. Different capitals at the same time represent the difference was extremely ($P < 0.01$), different small letters represent the difference was stastically ($P < 0.05$)

1017 nt) of PoRV and the S gene (1471–2415 nt) of PEDV become the targets of the co-expression of eukaryotic plasmids. Besides, Linker engineering is important for construction of recombinant fusion proteins which can adopt loop conformation to relax the intramolecular strain between the two half-molecules [12, 24]. In this study, Linker engineering was introduced between PoRV VP7 gene and PEDV S gene. Bioinformatics analysis found that the basic biological activities of VP7 gene and S gene in fusion gene kept unchanged, which indicated that linker would not affect the protein structure and biological function. Then, we successfully constructed PoRV VP7 gene (49–1017 nt), Linker engineering, and PEDV S gene (1471–2415 nt) co-expression recombinant plasmid pPI-2.EGFP.VP7.S. Western Blot results indicated that the recombinant plasmid pPI-2.EGFP.VP7.S can express in vivo.

To evaluate the immune effect induced by the DNA vaccine and confirm the optimal approach and dose, we analyzed the changes of specific serum antibody and non-specific parameters in different approaches and doses vaccinated mice. The results showed that eliciting powerful and long-lasting humoral and cellular immune response can be induced in vaccinated mice. The immune effect of intramuscular injection was better than subcutaneous injection. This phenomenon could be due to the structure of muscle cell, belonging to multinucleated cells that have plenty of sarcoplasmic reticulum and the transverse tubular system containing extracellular fluid, and deeply wear into the muscle cells [2]. So muscle cells are suitable for the intake and expression of DNA. The levels of PEDV and PoRV IgG in mice serum of high dose group (200 μg) were significantly higher than that of middle (100 μg) and low (50 μg) dose groups ($P < 0.05$). IFN-γ, IL-4, and T lymphocyte proliferation induced by high-dose group were higher than middle- and low-dose groups. The results confirmed that compared to 100 μg and 50 μg, the immune effect of 200 μg pPI-2.EGFP.VP7.S was better. The results were consistent with the report of Belperron A A ect [1].

In order to enhance the immunization of pPI-2.EGFP.VP7.S, we studied the effects of co-immunization with pPI-2.EGFP.VP7.S and IFN-α, IL-12, and pig spleen transfer factor in mice, respectively. The results showed that three kinds of adjuvants induced high levels of the PoRV and PEDV specific IgG, and the IFN-α exhibited best activity. The levels of IFN-γ and IL-4 and Spleen lymphocyte proliferation

Table 2 Cellular immune responses of the mice immunized with different doses, different approaches, and different adjuvant

Groups	OD_{450}	IFN-γ concentration (pg/mL)
a.		
A	$0.295^{Cc} \pm 0.0211$	$89.0741^{Cc} \pm 6.3730$
B	$0.356^{Aa} \pm 0.0254$	$134.2593^{Aa} \pm 9.5688$
C	$0.272^{Dd} \pm 0.0227$	$72.0370^{Dd} \pm 6.0216$
D	$0.255^{Ee} \pm 0.0231$	$59.4444^{Ee} \pm 5.3704$
E	$0.349^{ABa} \pm 0.0317$	$129.0741^{ABa} \pm 11.7340$
F	$0.303^{BCc} \pm 0.0217$	$95.0000^{BCc} \pm 6.7963$
G	$0.321^{Bb} \pm 0.0229$	$108.3333^{Bb} \pm 7.7116$
H	$0.202^{Ff} \pm 0.0224$	$20.1852^{Ff} \pm 2.2428$
K	$0.200^{Ff} \pm 0.0222$	$18.7037^{Ff} \pm 2.0864$
I	$0.369^{Aa} \pm 0.0194$	$143.8889^{Aa} \pm 7.5770$
J	$0.361^{Aa} \pm 0.0201$	$137.9630^{Aa} \pm 7.6687$
Groups	OD_{450}	IL-4 concentration (pg/mL)
b.		
A	$0.409^{DEd} \pm 0.0292$	$44.5855^{DEd} \pm 3.1763$
B	$0.537^{Bb} \pm 0.0566$	$74.5621^{Bb} \pm 7.8560$
C	$0.352^{Ee} \pm 0.0371$	$31.2365^{Ee} \pm 3.2881$
D	$0.335^{Ee} \pm 0.0353$	$27.2553^{Ee} \pm 2.8788$
E	$0.471^{Cc} \pm 0.0336$	$59.1054^{Cc} \pm 4.2168$
F	$0.424^{Dd} \pm 0.0447$	$48.0984^{Dd} \pm 5.0728$
G	$0.433^{CDd} \pm 0.0456$	$50.2061^{CDd} \pm 5.2922$
H	$0.281^{Ff} \pm 0.0296$	$14.6089^{Ff} \pm 1.5402$
K	$0.288^{Ff} \pm 0.0303$	$16.2482^{Ff} \pm 1.7054$
I	$0.618^{Aa} \pm 0.0441$	$93.5316^{Aa} \pm 6.6741$
J	$0.597^{ABa} \pm 0.0427$	$88.6136^{ABa} \pm 6.3329$

Serum samples were collected at 42 dpi to determine the cytokines IFN-γ (**a**) and IL-4 (**b**). Different capitals represent the difference was extremely significant ($P < 0.01$), different small letters represent the statistically difference was stastically significant ($P < 0.05$)

of three kinds of adjuvants were significantly higher than those of intramuscular injection group ($P < 0.01$). The effect of IFN-α as vaccine adjuvant has been discussed a lot over the years. IFN-α can exert its antiviral effect by stimulating the body to produce anti-viral proteins and inducing the body to produce adaptive immune response to eliminate invading pathogens. Meanwhile, IFN-α associates with the production of antibody. It is reported that IFN-I is the necessary signal of B cell differentiating into plasma cell. Although there lack of evidence of IFN-I can produce antigen specific B cells, B cells cannot be further activated and produce neutralizing antibody [10]. Cheng G et al. found that IFN-α induced strong induction of FMDV-specific neutralizing antibody and significant T cell-mediated immune responses through IFN-a as an adjuvant for a recombinant FMD protein vaccine [3]. The ability of IL-12 to induce protective immunity through enhancing proliferation of T cells and NK cells, increasing cytolytic activities of T cells, NK cells, and macrophages, activating T helper 1 (Th1) cells, and inducing production of IFN-γ and other cytokines [13]. Transfer factor can activate T lymphocytes and enhance the cellular immunity and humoral immunity. Wang R et al. found that transfer factor of pig spleen could increase cellular immunity function through inactivated porcine parvovirus oil emulsion vaccine by co-inoculating porcine transfer factor in mice, which had wide development value as immune adjuvant [20]. With the restricting of antibiotics, transfer factor will have advantage of epidemic disease preventing and curing on livestock and poultry.

In summary, a recombinant plasmid pPI-2.EGFP.VP7.S was constructed based on the dominant antigen genes of PEDV S gene and PoRV VP7 gene as vaccine. The effect of the DNA vaccine was evaluated in mice. The DNA vaccine pPI-2.EGFP.VP7.S showed a well immunogenicity through adjust dose, approach, and adjuvant in eliciting a humoral

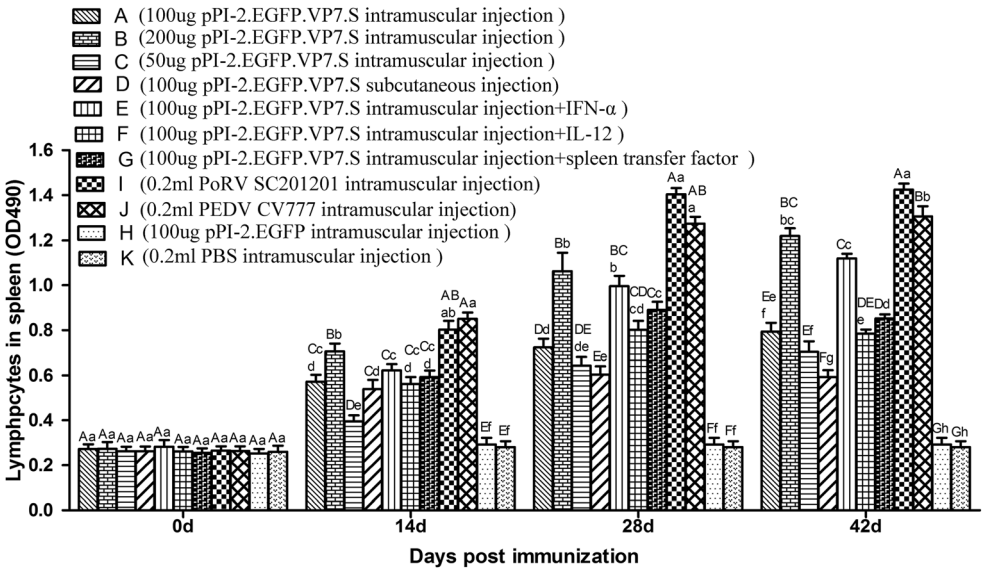

Fig. 6 The mice were immunized with different doses, approaches, and adjuvant. In addition, the empty vector pPI-2.EGFP, PBS, PoRV, and PEDV were used as controls. The proliferation of mouse spleen lymphocytes.was analyzed by MTT assays with ConA. The *y*-axis represents the lymphocyte proliferation levels (OD490). Different capitals at the same time represent the difference was extremely ($P < 0.01$), different small letters represent the difference was stastically ($P < 0.05$)

and cellular responses against PoRV and PEDV. Results showed the feasibility of using pPI-2.EGFP.VP7.S as a candidate DNA vaccine for PoR and PED.

Funding information This work was supported by the Science and technology program of Sichuan (Project No. 2014NZ0043) (Project No. 2017NZ0038), Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (2015BAD12B04-2.3), and Sichuan Crops and Animals Breeding Special Project during the Thirteenth Five-year Plan Period (2016NYZ0052).

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Belperron AA, Feltquate D, Fox BA, Horii T, Bzik DJ (1999) Immune responses induced by gene gun or intramuscular injection of DNA vaccines that express immunogenic regions of the serine repeat antigen from *Plasmodium falciparum*. Infect Immun 67: 5163–5169
2. Chen HW, Pan CH, Liau MY, Jou R, Tsai CJ, Wu HJ, Lin YL, Tao MH (1999) Screening of protective antigens of Japanese encephalitis virus by DNA immunization: a comparative study with conventional viral vaccines. J Virol 73:10137–10145
3. Cheng G, Zhao X, Yan W, Wang W, Zuo X, Huang K, Liu Y, Chen J, Wang J, Cong W (2007) Alpha interferon is a powerful adjuvant for a recombinant protein vaccine against foot-and-mouth disease virus in swine, and an effective stimulus of in vivo immune response. Vaccine 25:5199–5208
4. Gabbay YB, † AAB, Oliveira DS, Linhares AC, Mascarenhas JDP, Barardi CRM, Simões CMO, Wang Y, Glass RI, and Jiang B. Evidence for zoonotic transmission of group C rotaviruses among children in Belém, Brazil. J Med Virol 2008, 80, 1666–1674
5. Gao Y, Pei C, Sun X, Zhang C, Li L, Kong X (2018) Plasmid pcDNA3.1-s11 constructed based on the S11 segment of grass carp reovirus as DNA vaccine provides immune protection. Vaccine 36: 3613–3621
6. Guo XQ, Wang LQ, Qiao H, Yang XW, Yang MF, Chen HY (2015) Enhancement of the immunogenicity of a porcine circovirus type 2 DNA vaccine by a recombinant plasmid coexpressing capsid protein and porcine interleukin-6 in mice. Microbiol Immunol 59:174–180
7. Homwong N, Diaz A, Rossow S, Ciarlet M, Marthaler D (2016) Three-level mixed-effects logistic regression analysis reveals complex epidemiology of swine rotaviruses in diagnostic samples from North America. PLoS One 11:e0154734
8. Huang KY, Yang GL, Jin YB, Liu J, Chen HL, Wang PB, Jiang YL, Shi CW, Huang HB, Wang JZ (2017) Construction and immunogenicity analysis of lactobacillus plantarum expressing a porcine epidemic diarrhea virus S gene fused to a DC-targeting peptide. Virus Res
9. Karlsson I, Borggren M, Rosenstierne MW, Trebbien R, Williams JA, Vidal E, Vergara-Alert J, Foz DS, Darji A, Sisteré-Oró M (2018) Protective effect of a polyvalent influenza DNA vaccine in pigs. Vet Immunol Immunopathol 195:25–32
10. Kim D, Martinezsobrido L, Choi C, Petroff N, Garcíasastre A, Niewiesk S, Carsillo T (2011) Induction of type I interferon secretion through recombinant Newcastle disease virus expressing measles virus hemagglutinin stimulates antibody secretion in the presence of maternal antibodies. J Virol 85:200–207
11. Lee S, Kim Y, Lee C (2015) Isolation and characterization of a Korean porcine epidemic diarrhea virus strain KNU-141112. Virus Res 208:215–224
12. Li G, Huang Z, Zhang C, Dong BJ, Guo RH, Yue HW, Yan LT, Xing XH (2016) Construction of a linker library with widely controllable flexibility for fusion protein design. Appl Microbiol Biotechnol 100:215–225
13. Metzger DW (2010) Interleukin-12 as an adjuvant for induction of protective antibody responses. Cytokine 52:102–107
14. Molinari BL, Possatti F, Lorenzetti E, Alfieri AF, Alfieri AA (2016) Unusual outbreak of post-weaning porcine diarrhea caused by single and mixed infections of rotavirus groups A, B, C, and H. Vet Microbiol 193:125–132
15. Park JE, Shin HJ (2014) Porcine epidemic diarrhea virus infects and replicates in porcine alveolar macrophages. Virus Res 191:143–152
16. Pensaert MB, De BP (1978) A new coronavirus-like particle associated with diarrhea in swine. Arch Virol 58:243–247
17. Qiao X, Li G, Wang X, Li X, Min L, Li Y (2009) Recombinant porcine rotavirus VP4 and VP4-LTB expressed in Lactobacillus casei induced mucosal and systemic antibody responses in mice. BMC Microbiol 9:249
18. Suocheng W, Tuanjie C, Changjun S, Fengling T, Zhongren M (2015) Subunit vaccine preparation of bovine rotavirus and its efficacy in mice. Iran J Immunol 12:188
19. Wang L, Byrum B, Zhang Y (2014) New variant of porcine epidemic diarrhea virus, United States, 2014. Emerg Infect Dis 20: 917–919
20. Wang RN, Wang YB, Geng JW, Guo DH, Liu F, Chen HY, Zhang HY, Cui BA, Wei ZY (2012) Enhancing immune responses to inactivated porcine parvovirus oil emulsion vaccine by co-inoculating porcine transfer factor in mice. Vaccine 30:5246–5252
21. Yang WY, Zhu L, Zhou YC, Guo WZ, Zhi-Wen XU, Center AB, University SA (2014) Isolation and identification of porcine rotavirus in Sichuan province and its proliferation profile detected by fluorescence quantitative PCR. Chin J Vet Sci 34:192–198
22. Ying W, Zhang B, Li J, Aipire A, Li Y, Zhang F (2018) Enhanced contraception of canine zona pellucida 3 DNA vaccine via targeting DEC-205 in mice. Theriogenology 113:56–62
23. Zhang Y, Zhang X, Liao X, Huang X, Cao S, Wen X, Wen Y, Wu R, Liu W (2016) Construction of a bivalent DNA vaccine co-expressing S genes of transmissible gastroenteritis virus and porcine epidemic diarrhea virus delivered by attenuated Salmonella typhimurium. Virus Genes 52:354–364
24. Zhao HL, Yao XQ, Xue C, Wang Y, Xiong XH, Liu ZM (2008) Increasing the homogeneity, stability and activity of human serum albumin and interferon-alpha2b fusion protein by linker engineering. Protein Expr Purif 61:73–77
25. Zhao Z, Liu HL, Fan GU, Zhou TF, Qin JJ, Zhi-Wen XU, Center AB, and University SA (2015) Part characteristics of porcine epidemic diarrhea virus SC-P strain. Chin J Vet Sci