Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Research in Veterinary Science 144 (2022) 34–43

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Expression and immunogenicity of nsp10 protein of porcine epidemic diarrhea virus

Liting Zhu [a], Shiguo Liu [a], Zewen Zhuo [a], Yanxi Lin [a], Yanni Zhang [b], Xiaoling Wang [a], Lingbao Kong [a], Ting Wang [a,*]

[a] Institute of Pathogenic Microorganism, College of Bioscience and Engineering, Jiangxi Agricultural University, Nanchang, Jiangxi, China
[b] Jiangxi Province Center for Disease Control and Prevention, Nanchang, Jiangxi, China

ARTICLE INFO

Keywords:
Porcine epidemic diarrhea virus
nsp10
Immunity

ABSTRACT

Porcine epidemic diarrhea virus (PEDV), a swine enteropathogenic coronavirus, causes lethal watery diarrhea to the piglets, which poses significant economic losses and public health concerns. The nsp10 protein of PEDV is essential regulatory subunits that are critical for virus replication. Since PEDV nsp10 is a crucial regulator of viral RNA synthesis, it is promising that nsp10 might become anti-virus drugs target or candidate for rapid diagnosis of PEDV infection. In this study, the PEDV nsp10 was inserted into pMAL-c2x-MBP / pET-28a vector, efficiently and stably expressed in *E.coli* system. Then the purified nsp10 protein was found to mediate potent antibody responses in immunized mice. The antibodies of immunized mice and PEDV infection swine strongly recognized purified nsp10 protein from cell lysates. Furthermore, cytokines test revealed that the expression of IL-2, IL-4, IL-10, TNF-α, IFN-γ were significantly higher than those in control group, indicated that purified nsp10 protein induce the cellular immune response mechanism in mice. Using modified seroneutralization test, we also demonstrated that sera from nsp10-immunized mice inhibited PEDV replication to some extent. These findings suggest that nsp10 has a high immunogenicity. This study may have implications for future development of PEDV detection or anti-virus drugs for swine.

1. Introduction

Porcine epidemic diarrhea (PED) is a highly contagious viral disease in pigs caused by porcine epidemic diarrhea virus (PEDV) that characterized by severe diarrhea, vomiting, and dehydration with a high mortality in piglets and brought substantial economic losses(Pensaert and Martelli, 2016; Sun et al., 2012; Wang et al., 2016). In China, PEDV was first identified in the 1980s, and in 2010, a large-scale outbreak of PED occurred in China, causing tremendous economic losses(Li et al., 2012; Sun et al., 2012; Tian et al., 2014). PEDV belongs to the order *Nidovirales*, family *Coronaviridae* and genus *Alphacoronavirus* and is an envelop virus with a single-stranded, positive-sense RNA genome. The genome of PEDV is approximately 28 kb and consist of the 5′ untranslated region (5' UTR), 3′ poly (A) tail, seven open reading frames (ORFs) which include ORF1a, ORF1b, S, ORF3, E, M and N genes(Duarte et al., 1993; Song and Park, 2012). The ORF1a and ORF1b encode two large replicase polyprotein (pp1a and pp1ab), which are subsequently processed into 16 nonstructural proteins (nsp1 to 16)(Subissi et al., 2014).

Nsp10 protein is existent exclusively in CoVs so far which is a zinc-finger protein through detecting the crystal structure of SARS CoV nsp10 protein(Joseph et al., 2006). Crystallographic or nuclear magnetic resonance structures have shown that nsp10 have the ADP-ribose 1-phosphatase (ADRP) activity and RNA binding activity(Anand et al., 2002). Nsp10 is a crucial regulator involved in viral RNA synthesis and is necessary for viral replication via regulating the nsp14 ExoN and nsp16 2'-O-MTase activities(Bouvet et al., 2010; Bouvet et al., 2012; Donaldson et al., 2007b). The nsp16 *S*-methyltransferase activity can only be activated when combined with nsp10 as dimer structure(Decroly et al., 2011). In addition, nsp10 also forms a complex with nsp14 to promote the cleavage effect of mismatched nucleotides by nsp14(Bouvet et al., 2012). Donaldson et al reported that mutant variant of nsp10 inhibits the main protease, 3CLpro, blocking its function completely at the nonpermissive temperature which implicate nsp10 as being a critical factor in the activation of 3CLpro function(Donaldson et al., 2007a). Furthermore, PEDV nsp10 enhances the inhibitory effect of nsp16 on IFN-β production, negatively regulates innate immunity to promote viral

* Corresponding author.
E-mail address: tingwang@jxau.edu.cn (T. Wang).

https://doi.org/10.1016/j.rvsc.2021.12.024
Received 8 April 2021; Received in revised form 1 December 2021; Accepted 28 December 2021
Available online 3 January 2022

Table 1
Primers used for Real-time PCR.

Primer	Sequence (5′ → 3′)	Fragment size (bp)
IL-2	GTGCTCCTTGTCAACAGCG GGGGAGTTTCAGGTTCCTGTA	171
IL-4	GGTCTCAACCCCCAGCTAGT GCCGATGATCTCTCTCAAGTGAT	102
IL-10	GCTCTTACTGACTGGCATGAG CGCAGCACTAGGAGCATGTG	105
TNF-α	GACGTGGAACTGGCAGAAGAG TTGGTGGTTTGTGAGTGGAG	228
IFN-γ	ATGAACGCTACACACTGCATC CCATCCTTTTGCCAGTTCCTC	182
GAPDH	AGGTCGGTGTGAACGGATTTG TGTAGACCATGTAGTTGAGGTCA	123
PEDV N	GGAGAATTCCCAAGGGCGAA ATCTGAGCATAGCCTGACGC	121

proliferation(Shi et al., 2019). Considering these biological features of the nsp10 protein, it would be an appropriate target for developing effective detection tool or drugs against PEDV.

In the study, nsp10 protein were constitutively expressed in *Escherichia coli* (BL21) and explored for its ability to induce immune responses. We found that nsp10 was capable of inducing an efficient antibody response in immunized mice and high expression of cytokines in lymphocytes of mouse spleen.

2. Materials and methods

2.1. Plasmid and animals

Prokaryotic expression vector pET-28a,pMAL-c2x-MBP;The competent cells *E.coli*-BL21(DE3), E.coli-DH5α are stored in our laboratory. Healthy Kunming female mice aged 6–8 weeks were purchased from Jiangxi University of Traditional Chinese Medicine. Porcine epidemic diarrhea virus strain CV777(GenBank: KT323979.1)are stored in our laboratory.

2.2. Main reagents

RNA extraction kit and SYBR Color qPCR Master Mix was purchased from VazymeBiotech Co.,Ltd. 2 K DNA Marker; 15 K DNA Marker; BamHI, *Xho*I, *Eco*RI, *Hin*dIII endonuclease; T4 DNA ligase; Taq DNA polymerase and PrimeScript 1st strand cDNA Synthesis Kit were purchased from Takara Bio company. Plasmid extraction kit was purchased from TIANGEN Biotech Co.,LTD. Urea was purchased from Xilong Chemical.14-100 kDa Protein Marker; 10-180 kDa Protein Marker; 5× SDS-PAGE Loading Buffer were purchased from TransGen Biotech. PVDF membrane was purchased from Millipore of Sigma-Aldrich Company. 6× His-Tag Monoclonal antibodies, MBP Rabbit polyclonal antibodies, HRP- Goat anti Mouse IgG and HRP- Goat anti Pig IgG were purchased from Proteintech Group. The ELISA kit was purchased from Huamei Biological Engineering Co. Ltd. NTA Agarose was acquired from QIAGEN of Sigma-Aldrich Company. Freund's complete adjuvant and Freund's incomplete adjuvant was purchased from ThermoFisher Scientific.

2.3. PEDV nsp10 Bioinformatics Analysis

The prediction of nsp10 immune epitopes were performed using the predictor of Bepipred on the IEDB website (http://tools.immuneepitope.org/main/). The putative three-dimension of nsp10 protein was generated using the software Swiss-Model(https://swissmodel.expasy.org/).

2.4. Plasmid construction

Total RNA Extraction Reagent (Vazyme Biotech, Nanjing) was used to isolate the total RNA from PEDV-infected vero cells according to the manufacturer's instructions. The nsp10 was amplified from total RNA of infected cells according to the nsp10 specific primers designed by PEDV strain CV777 (GenBank accession number KT323979.1). The nsp10 genes were separately inserted into the pET-28a vector and pMAL-c2x-MBP vector. The inserted gene fragments of constructed plasmid were confirmed by sequencing.

2.5. Expression and purification of recombinant protein

The recombinant plasmids (pMAL-c2x-MBP-nsp10, pET-28a-nsp10) were transformed into *E.coli* BL21 (DE3) strain separately. The expression of these proteins was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG). After induction, the expression levels in the whole cell lysate, the supernatant and the sediment were examined by SDS-PAGE gel. Purification of recombinant proteins was performed as described previously(Wang et al., 2009). Briefly, cells were pelleted and suspended in 8 M urea buffer, followed by centrifugation at 12,000*g*, 4 °C for 30 min. The supernatant was applied to Ni–NTA affinity column. Purified proteins were eluted with buffers containing different concentrations of imidazole. Samples were stored at −80 °C for further use.

2.6. Immunoblotting

Immunoblotting was performed as described previously. Briefly, protein samples were separated by SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After protein transfer, the membranes were blocked for 1 h with 10% nonfat dry milk. The blots were then incubated with a primary antibody at 4 °C overnight. The primary antibodies could be the antisera from mice immunized with the nsp10 protein, antisera from healthy or PEDV-infected swine; commercial antibodies against His-tag, or MBP-tag. The membranes were then incubated with HRP-conjugated secondary antibodies: goat anti-mouse or -swine. Finally, the proteins were visualized with Clarity ECL Immunoblotting substrate (Bio-Rad).

2.7. Mouse immunization and ethics

Kunming mice were randomly assigned to the following groups and immunized with 50 μg of the recombinant proteins in the presence of complete Freud's adjuvant (subcutaneous). The mice were subsequently boosted by the same method twice at one-week intervals with incomplete Freud's adjuvant in the subcutaneous regimen. Blood was harvested at different time-points. Mice immunized with PBS served as negative controls. Mouse physiological Status were monitored daily for the following three weeks.

Mouse experiments were approved by Animal Care and User Committee and Laboratory Animal Ethics Committee at Jiangxi Agricultural University and performed under the approved guidelines.

2.8. Recombinant protein-coated ELISA

ELISA plates were coated overnight at 4 °C with 50 ng/well of each purified recombinant protein or the cell lysate dissolved in coating buffer (0.016 M Na_2CO_3, 0.034 M $NaHCO_3$, pH 9.6) followed by the blocked with 5% non-fat milk for one hour at room temperature. After extensive wash with PBST, mouse serum samples were added to wells and incubated at 37 °C for 1 h. After extensive wash, HRP-conjugated goat anti-mouse antibody was added to the wells for one hour-incubation at room temperature. Finally, substrates were added to the plate. The absorbance of each well was measured with a Bio-Rad microplate reader at a wavelength of 450 nm.

2.9. Cytokine profiling in serum and splenocytes

Sera and splenocytes were collected from protein-immunized mice.

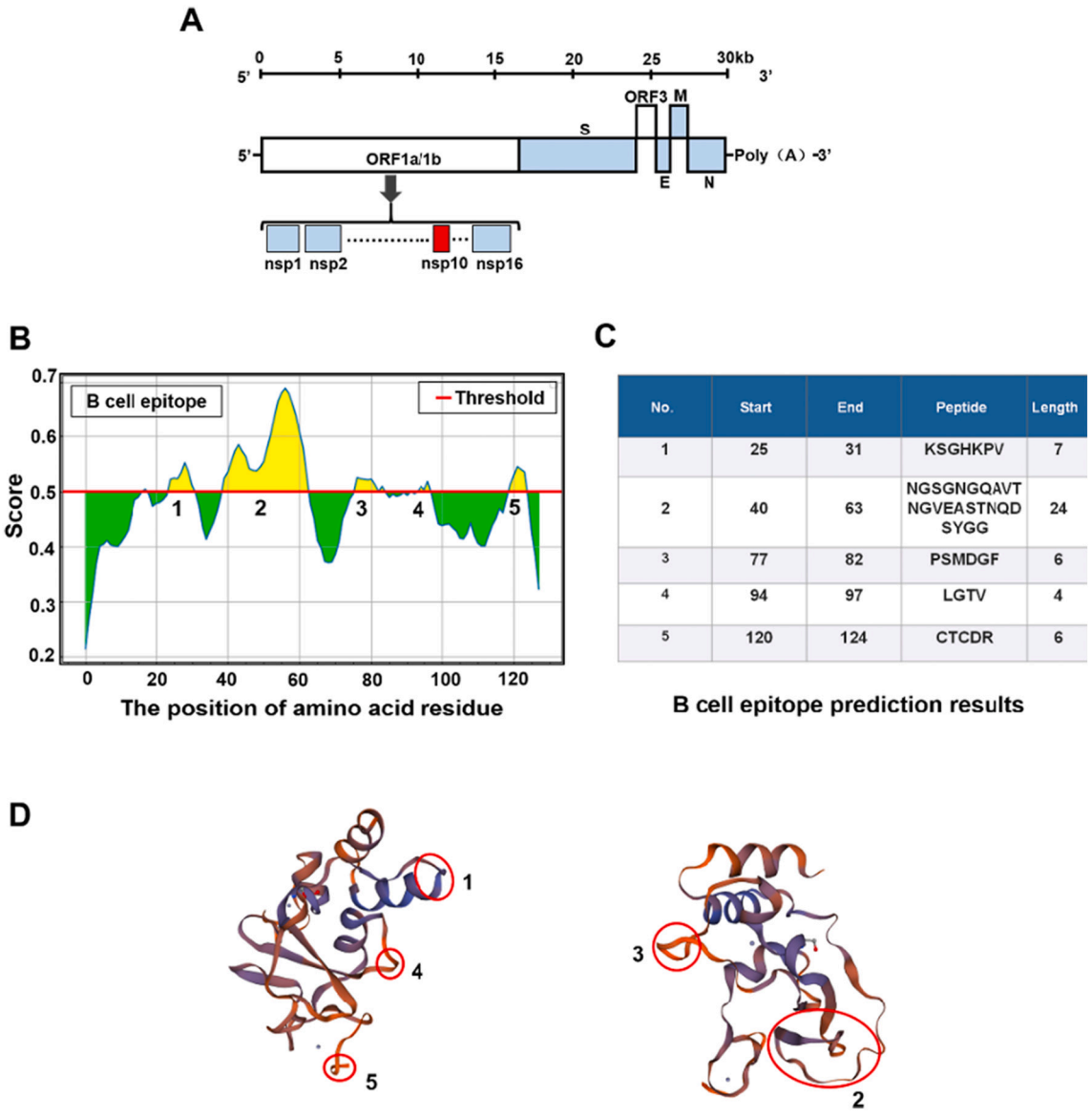


Fig. 1. Schematic diagram of PEDV genome structure and nsp10 protein three-dimensional (3D) structure and prediction of possible antigenic epitopes. (A) Schematic diagram of PEDV genome. (B) Prediction of B-cell potential antigenic epitopes using BepiPred with the threshold value ≥0.5. (C) The amino acid residues of B cell antigen epitope. (D) The structure of nsp10 predicted by Swiss-model and marked with antigenic epitopes.

Cytokines including IL-4, TNF-α in serum samples and supernatant from cells treated with purified nsp10 protein were measured by ELISA as described previously. Briefly, the harvested sera were added to the pre-coated ELISA plate. After incubation for one hour at room temperature, 50 μl of biotinylated antibody was added to each well and incubated for 90 min at 37 °C. After 4 times of wash, 100 μl of streptavidin-HRP was added to each well. The plates were sealed and incubated at 37 °C for 30 min. After adding substrate for development, the absorbance was recorded at 450 nm. The mRNA expression of Cytokines including IL-2, IL-4, IL-10, TNF-α, IFN-γ in splenocytes were detected by Real-time PCR as described previously(Matsuda, 2017). In brief, Gene expressions were quantified using TB Green®Premix Ex Taq™II (Takara Biotechnology, China) and the qRT-PCR was performed in an Applied Biosystems® 7500 Fast Real-time System. The primers were specific designed using Primer Premier 5.0 (Table 1).

2.10. Seroneutralization test and Real-time quantitative RT-PCR

The seroneutralization experiment was proceeded as previously described with minor modifications(Gauger and Vincent, 2014; Li et al., 2020). Vero cells were seeded with 5×10^4 cells per well into 96-well plates at least 6 h prior to infection. The test samples were serially diluted 2-fold from 1:2 to 1:256 with DMEM, eight wells were used for each dilution. Then the serial dilution of sera samples were applied to cells and incubated for 1 h at 37 °C. After that, the sera samples were removed and cells incubated with 200 $TCID_{50}$ of PEDV at 37 °C for 1 h. The cytopathic effect was examined for 2 days post-infection. The wells infection with 200 $TCID_{50}$, 20 $TCID_{50}$, 2 $TCID_{50}$ of PEDV were considered as virus-infection control.

For standardization of the absolute quantification assay, the cDNA of PEDV N gene was inserted into pCAGGA-HA vector and the plasmid DNA isolated from positive clone DH5α. qRT-PCR was carried out in a 20-μl reaction mixture with 2 μl cDNA or standard plasmid, 10 μl 2 × SYBR qPCR Master mix, 0.2 μM forward and reverse primers. Amplification and detection were performed in Applied Biosystems® 7500 Fast Real-time System. The specific primers of PEDV N are listed in Table 1.

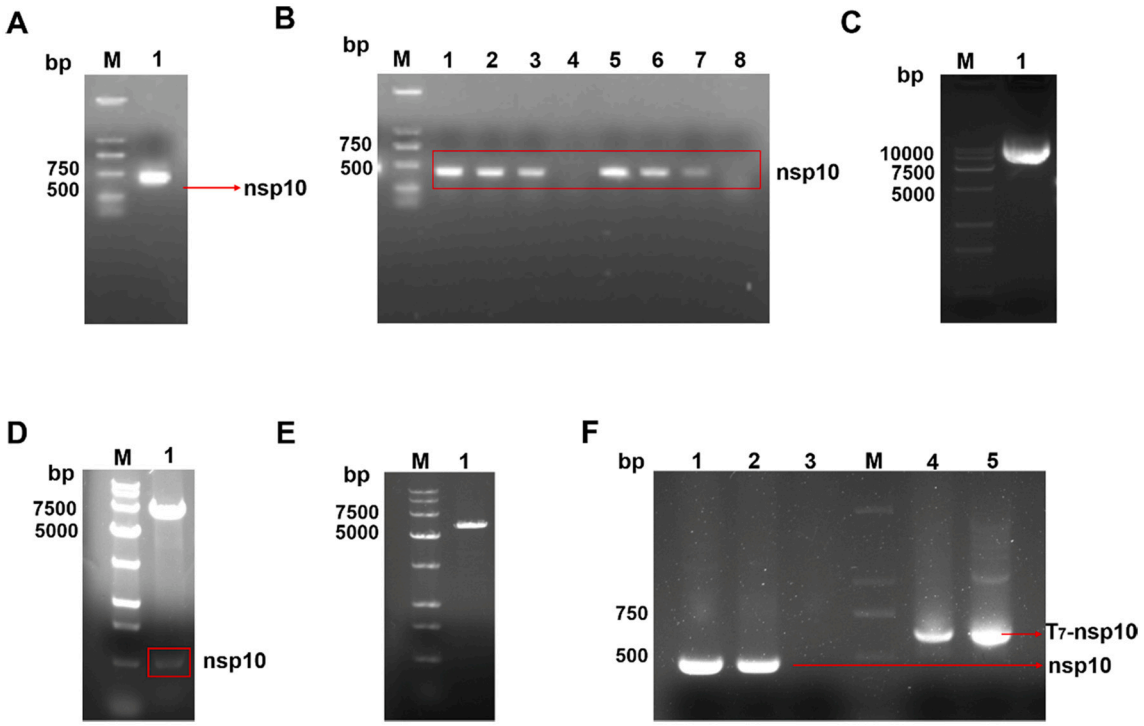


Fig. 2. Cloning and verification of PEDV nsp10 gene by PCR using specific primers or double enzyme digestion.
(A) The amplification of PEDV nsp10 by PCR. (B) The identification of positive clone after plasmid transformation by bacterial PCR. Lane 1 to lane 7 were samples and lane 8 was negative control. (C) The identification of pMAL-c2x-MBP-nsp10 after plasmid extraction from positive clone. (D) Recombinant plasmid pMAL-c2x-MBP-nsp10 was identified by double enzyme digestion. (E) The identification of pET-28a-nsp10 after plasmid extraction from positive clone. (F) The recombinant plasmid pET-28a-nsp10 was identified by PCR, lane 1 and lane 2 were nsp10 amplification, lane 4 and lane 5 were nsp10 amplification by using the T7 primer, lane 3 was negative control.

2.11. Statistical analysis

Data analysis was performed using GraphPad Prism 8.0 software to determine statistical significance. Statistical differences between two groups were tested with Student's *t*-test. For all the tests, $P < 0.05$ was regarded as the statistical significance.

3. Results

3.1. Prediction of potential antigenic epitopes and three-dimensional (3D) structure of PEDV nsp10 protein

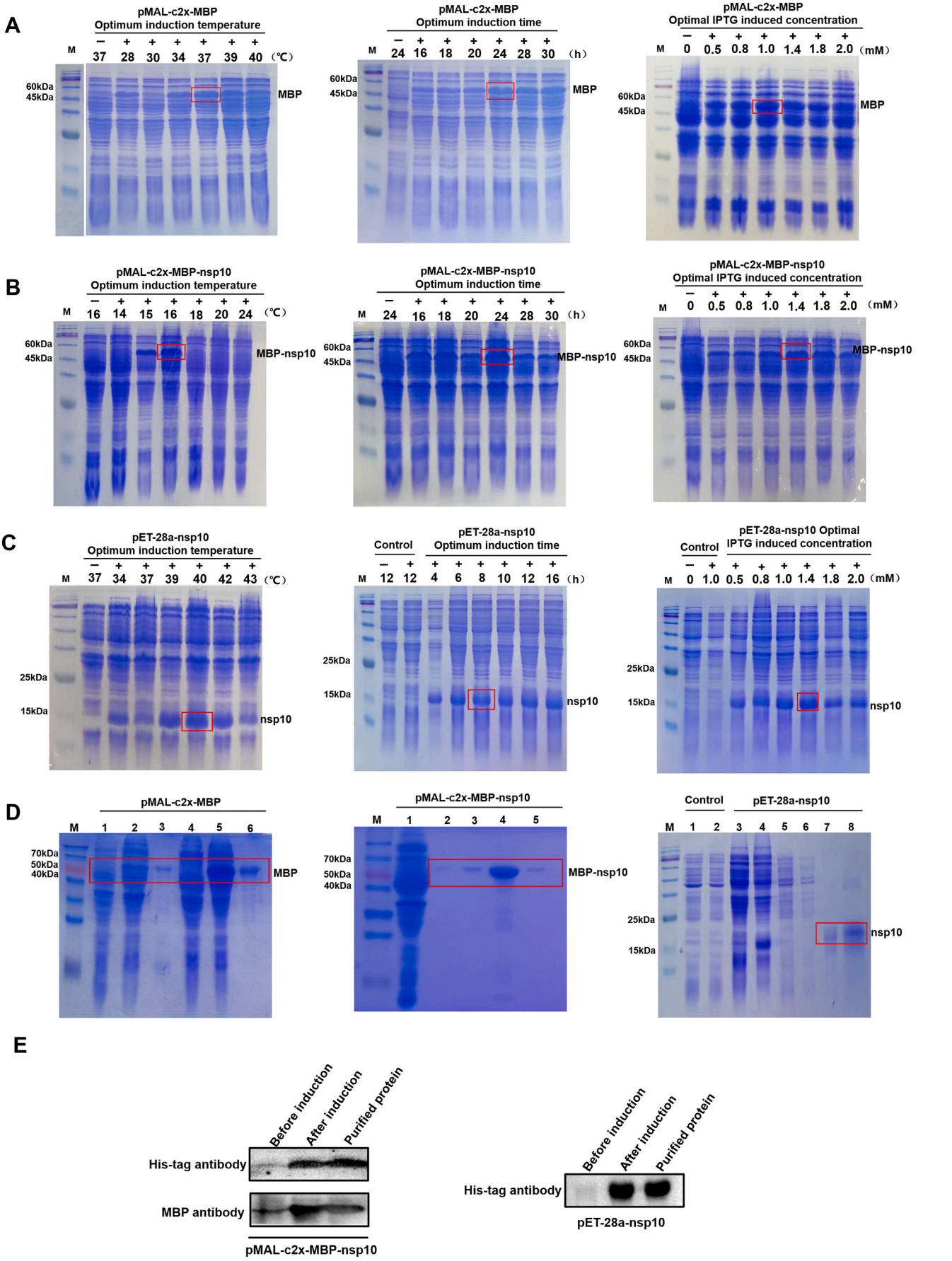
Schematic diagram of the genome of PEDV is shown in Fig. 1A. PEDV nsp10 protein sequence was subjected to Bepipred and Swiss-Model to forecast its linear B cell epitopes and 3D structure, respectively. Using a threshold value 0.5 for Bepipred, 5 potential B cell epitopes were found (Fig. 1B). It is speculated that main B cell antigen epitope of nsp10 may appear in the 25 to 31, 40 to 63, 77 to 82, 94 to 97, 120 to 124 amino acid residues (Fig. 1C). B cell antigen epitopes are shown in the Swiss-modeled nsp10 protein structure (Fig. 1D). Altogether, these results indicate that PEDV nsp10 protein may harbor potential for inducing host immune responses.

3.2. Construction of recombinant prokaryotic expression plasmid

By using PEDV cDNA as template, specific primers were designed for PCR amplification. The fragment of 408 bp was identified by 1.5% agarose gel electrophoresis, which was consistent with the expected size (Fig. 2A). After PEDV nsp10 gene was ligated with prokaryotic expression vector pET28a, pMAL-c2x-MBP by double digestion of BamH I, EcoR I, *Xho* I, Hind III, the ligation product was transformed into *E. coli* competent cell DH5α. A number of positive clones were randomly selected for PCR identification, and the amplified fragment was 408 bp (Fig. 2B). The correct clone strains identified by PCR were selected for plasmid extraction (Fig. 2C and E). The recombinant plasmid pMAL-c2x-MBP-nsp10 was identified by double enzyme digestion (Fig. 2D). Then the recombinant plasmid pET28a-nsp10 was identified by PCR using nsp10 specific primer and T7 primer respectively (Fig. 2F). The positive clone was sent to Sangon for sequencing. The sequence of PEDV nsp10 was identified based on a multiple alignment of PEDV CV777 strain. The sequence of positive clone was confirmed through DNAMAN sequence comparison. The recombinant expression vector pMAL-c2x-MBP-nsp10, pET28a-nsp10 was successfully constructed.

3.3. Expression and purification of recombinant PEDV nsp10 protein

To obtain nsp10 protein, the recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells, positive clones were randomly selected and expressed. The bacteria were collected and examined by SDS-PAGE gel, several positive clones were successfully expressed. The MBP protein in vector pMAL-c2x-MBP was successfully expressed, and the relative molecular weight of the MBP-nsp10 and nsp10 protein was 56kD and 16kD respectively. To obtain the optimal condition of protein expression, we tested different induction temperature (Fig. 3A left panel), induction time (Fig. 3A middle panel) and IPTG concentration (Fig. 3A right panel) on the expression level of MBP protein. Results indicated that the expression level of MBP was the highest at temperature 37 °C, induction time for 24 h and IPTG concentration for 1 mM. At the same time, we exploited different condition including different temperature (Fig. 3B left panel), different induction time (Fig. 3B middle panel) and different IPTG concentration (Fig. 3B right panel) to test the expression level of recombinant protein MBP-nsp10 protein. Results showed that the optimal condition of MBP-nsp10 expression was 16 °C for temperature, 24 h for induction time, 1.4 mM for IPTG



(caption on next page)

Fig. 3. Expression and purification of recombinant PEDV nsp10 protein.
(A-C) Expression of recombinant protein. *E.coli* cells transformed with plasmid of pMAL-c2x-MBP (A), pMAL-c2x-MBP-nsp10 (B) or pET-28a-nsp10 (C) were un-induced or induced with different temperature (left panel), different time (middle panel), and different IPTG concentration (right panel). After lysis, whole cell lysate was subjected to SDS-PAGE gel. *E.coli* cells transformed with an empty vector served as negative controls. The highlights indicated the optimum expression in different condition. (D) Purification of MBP, MBP-nsp10 and nsp10 protein. Left panel: MBP protein. Lane 1 and lane 4: un-induced cell lysates; lane 2 and lane 5: induced cell lysates; lane 3 and lane 6: elution fractions with imidazole of 250 mM. Middle panel: MBP-nsp10 protein. Lane 1: un-induced cell lysate; lane 2–5: elution fractions with imidazole of 100 mM, 200 mM, 250 mM, 300 mM. Right panel: nsp10 protein. Lane 1 and lane 2: un-induced or induced cell lysates transformed with plasmid of mock pET-28a; lane 3 and lane 4: un-induced or induced cell lysates transformed with plasmid of pET-28a-nsp10; lane 5 and lane 6: flow solution; lane 7 and lane 8: elution fractions with imidazole of 200 mM, 250 mM. (E) Identification of recombinant proteins by immunoblotting. Proteins were subjected to immunoblotting analysis using commercial anti-His tag antibody or anti-MBP tag antibody.

concentration. In addition, the best expression temperature of pET28a-nsp10 was at 40 °C (Fig. 3C left panel), and with the increase of temperature, the protein expression level showed a trend of first increasing and then decreasing. Then nsp10 protein was expressed in different concentration IPTG to confirm optimal concentration of IPTG. In summary, the best condition of pET28a-nsp10 expression was: 40 °C, 1.4 mM IPTG concentration (Fig. 3C right panel) induced by 8 h (Fig. 3C middle panel). The MBP, MBP-nsp10 and nsp10 protein was purified by Ni- column and eluted by 250 mM imidazole (Fig. 3D). To confirm the identities of *E.coli*-expressed recombinant proteins, we performed immunoblotting assays with the commercial anti-His tag antibody or anti-MBP antibody. Expected bands were detected for all recombinant proteins in IPTG-induced bacterial lysate samples, but not un-induced ones, demonstrating the identities of *E.coli*-expressed proteins (Fig. 3E).

3.4. Immunogenicity analysis of PEDV nsp10 protein in mice

After administration of nsp10 protein, antibody titers of mice sera were determined by ELISA. Sera of mice immunized with PBS solution was used as negative control. The results showed that antibody titers of nsp10 protein immunized mice were the highest at the fourth week, 1:43000 of MBP-nsp10 (Fig. 4A right panel) and 1:13000 of nsp10 (Fig. 4B left panel) respectively. In addition, we tested the antibody titers of MBP protein and found that the highest titer was at the third week, 1:31000. At the same time, we harvested sera from protein-immunized mice to examine the immunogenicity of these proteins. Immunoblotting results showed that mice sera specifically detected the corresponding recombinant proteins expressed in IPTG-induced transformed *E.coli*, but not in the un-induced samples (Fig. 4C). As a negative control, sera from mice treated with PBS did not recognize any recombinant proteins. We further evaluated the nsp10-specific immunogenicity with the sera from PEDV-infected pigs. The data from immunoblotting revealed that nsp10 recognized PEDV-positive pig sera but not PEDV-free pig sera (Fig. 4C right). Moreover, 30 sera samples from healthy or PEDV-infected pigs were collected, then investigated blindly by using commercial ELISA kit or nsp10 protein. The results showed that there is a high degree of consistency between PEDV ELISA kit and nsp10-ELISA (Fig. 4D). In summary, these data showed that the recombinant nsp10 protein expressed in *E.coli* retains the antigenicity that sera from PEDV-infected pigs can recognize.

3.5. Cellular responses analysis of PEDV nsp10 protein in mice

Cellular immune responses play an essential role in the defense against viruses(Benova et al., 2020). we examined levels of Th2 (IL-4) cytokines in mouse sera upon immunization with nsp10 protein. The results showed that the treatment of nsp10 protein, but not PBS, triggered mice to secrete cytokines substantially (Fig. 5A). After splenocytes were harvest from mice immunized with nsp10 protein and stimulated by purified nsp10 protein of different concentration in vitro, levels of Th1 (TNF-α) from cell supernatant were examined by ELISA after different incubation time. As shown in Fig. 5B, nsp10 protein triggered more secretions of cytokine TNF-α, compared with PBS treatment group. To further evaluate the cellular immunity at mRNA expression level, expression of cytokine IL-2, IL-4, IL-10, TNF-α, IFN-γ in splenocytes from immunized mice were detected by Real-time PCR assay. The relative expression levels of all genes were calculated by the $2^{-\triangle\triangle Ct}$ algorithm. The internal reference was GAPDH, which is a housekeeping gene. Compared with PBS immunized group, the expression level of cytokine in the nsp10-immunized group was significantly increased (Fig. 5C). All the results above collectively demonstrate that nsp10 is capable of initiating cellular immunity.

3.6. Mice serological protection assay and absolute PEDV quantitation

A seroneutralization test was performed to determine whether the sera antibody from mice immunized with nsp10 had protection activity. We incubated cells with serum dilutions (final dilution from 1:4 to 1:256) of immunized mice sera, then the cells were infected with 200 $TCID_{50}$ of PEDV. We observed the cytopathogenic effect for 2 days and the partial absence of CPE in >50% of the wells was defined as protection. As shown in Fig. 6A, 2^3 dilution of serum can block CPE in 50% of eight repetitions wells. The findings suggested that nsp10 antibodies may protect cells from PEDV infection. To further detect the absolute quantity of the viral load in serum-treated cells, the N gene expression of PEDV was detected by qRT-PCR as the plasmid pCAGGS-HA-N was used for the generation of a standard curve (Fig. 6B). The result showed that the copy numbers of PEDV RNA from nsp10 antibodies serum-treated cells were decreased compared with the PEDV-infection cells (Fig. 6C). The results presented here suggested that nsp10 antibodies from mice sera may inhibit PEDV replication to some extent.

4. Discussion

The coronavirus nsp10 contains two zinc-finger motifs(Matthes et al., 2006) and may function as part of a larger RNA-binding complex (Su et al., 2006). Previous researches have shown that coronavirus nsp10 is a critical multifunctional factor for activation of multiple replicative enzymes. Nsp10 molecules may thus serve as a platform for the recruitment of nsp14 or nsp16 to the replication-transcription complex in order to either stimulate nsp14 ExoN activity or activate nsp16 2-O-MTase activity(Snijder et al., 2016). Both nsp10-nsp14 and nsp10-nsp16 complexes may coexist which play essential role in different steps of viral RNA synthesis as part of larger nsp assembling factory(Bouvet et al., 2014). Considering its importance for virus replication, nsp10 represents an attractive target for anti-coronavirus

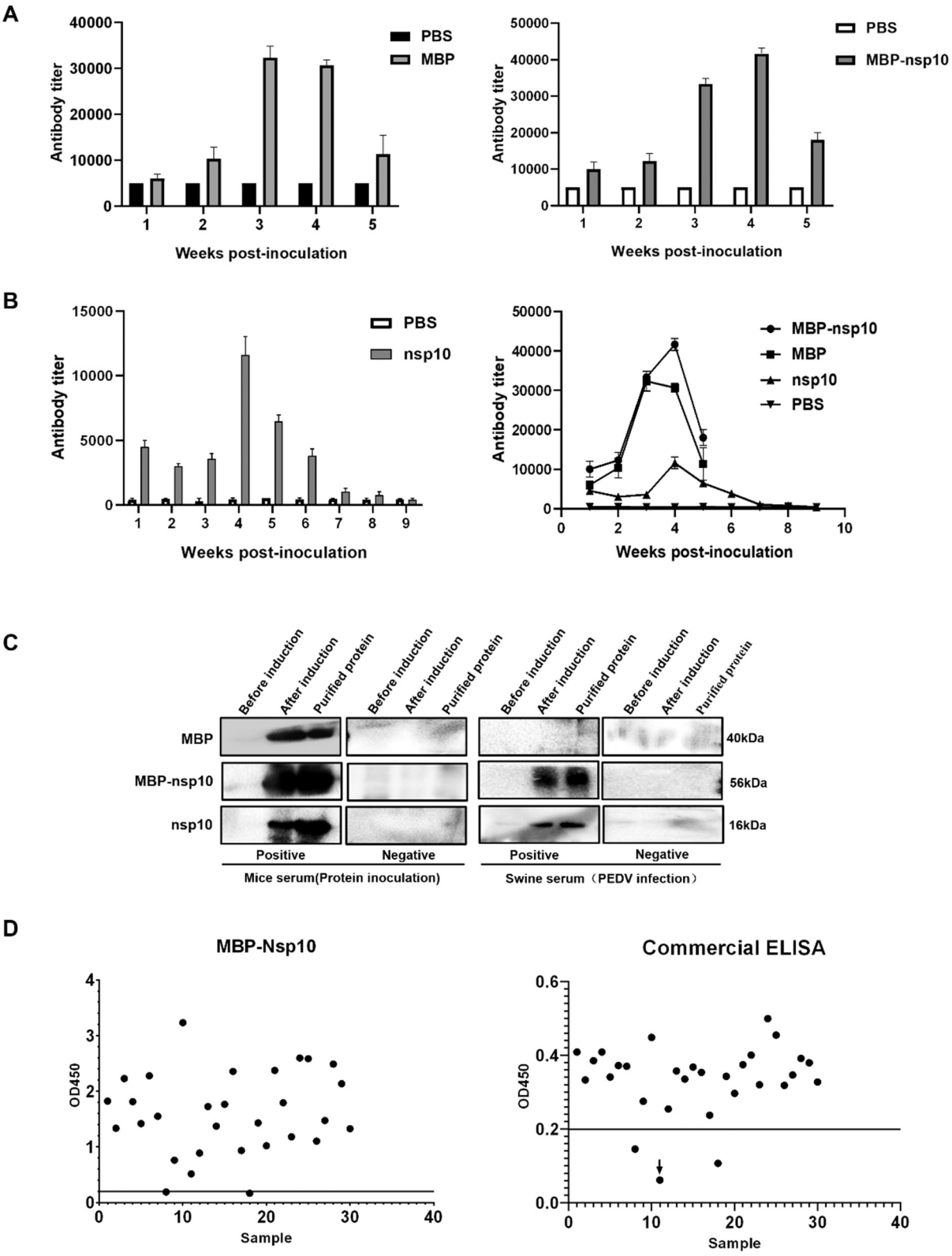


Fig. 4. Humoral responses induced by subcutaneous immunization of nsp10 recombinant protein in mice.
(A-B) KunMing mice (6-week old) were administrated subcutaneously with 50 μg of MBP, MBP-nsp10, nsp10. Antisera were collected and subjected to ELISA for detecting nsp10 antibody. (C) The lysates from un-induced or IPTG-induced cells and purified protein were used to react with corresponding protein-immunized mouse sera, PEDV-positive pig sera and control sera in the immunoblotting assays. (D) Comparison of nsp10-coated ELISA and commercial PEDV ELISA kit. Sera samples from PEDV-infected or mock-infected pig were test by nsp10-coated ELISA (left) and commercial ELISA kit (right). Each point represents a serum sample, and the line represents the cutoff value ($n = 30$).

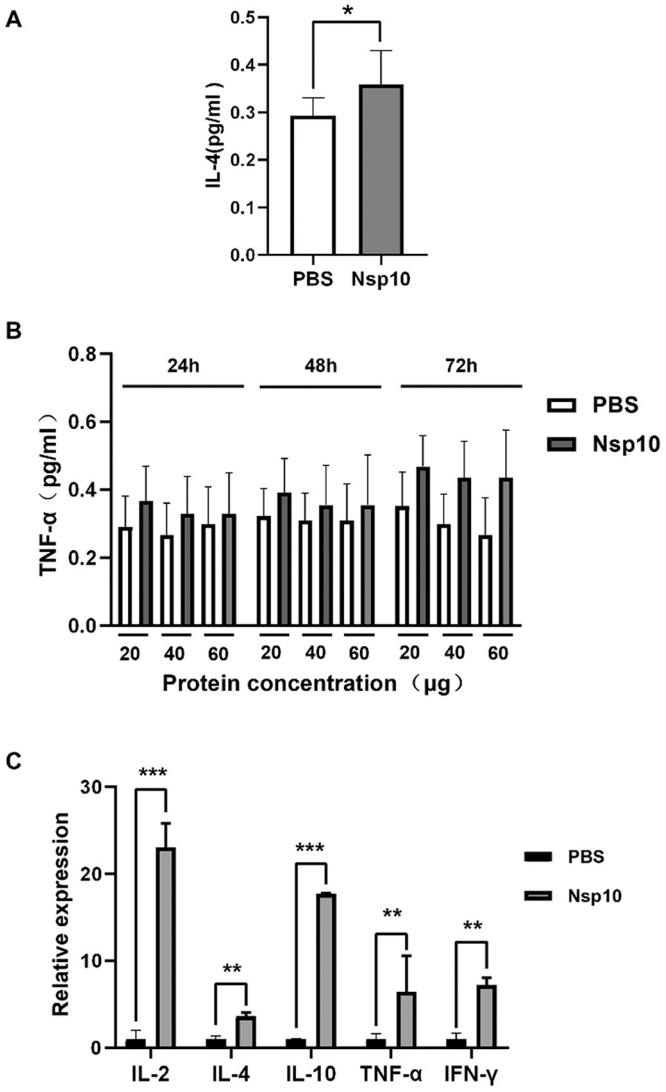


Fig. 5. The detection of cytokines in sera and spleen cells after subcutaneous immunization with nsp10 recombinant protein.
(A) Antisera from mice immunized with nsp10 protein were collected and subjected to ELISA for detecting cytokines (IL-4). (B) Splenocytes were harvested from immunized mice and incubated with 20 μg, 40 μg, 60 μg of nsp10 protein respectively. The culture medium was collected after incubation for 24 h, 48 h, 72 h, then detected cytokines (TNF-α). (C) The RNA of splenocytes from immunized mice was extracted and subjected to Real-time PCR for detecting the expression level of cytokines IL-2, IL-4, IL-10, TNF-α and IFN-γ.

drug development(Lin et al., 2020; Wang et al., 2015). Specific molecules or antibodies targeting the nsp10 and inhibiting the interaction with nsp14 and nsp16 might be applied to reduce or prevent virus proliferation(Ke et al., 2012). Because the nsp10 subunit is among the more conserved coronavirus proteins, such compounds or antibodies could be used in a prophylactic approach to prevent coronavirus infection just like the role of vaccine. The current focus of PEDV nsp10 research is on the interaction of nsp10 with nsp14 and nsp16 to regulate innate immunity(Lu et al., 2020; Shi et al., 2019). In the present study, the first aim was to stably express the full-length nsp10 protein of PEDV in *E.coli* system. Subsequently, we were able to successfully generate a stable *E.coli* system producing large amounts of nsp10 protein through optimization of expression conditions (Fig. 3A-C). Following the purification and concentration processes, nsp10 protein could be consistently harvested. Then mice were immunized with nsp10 protein and we determined whether or not they developed humoral immunity. Nsp10-specific antibodies were strongly detectable in mice sera collected from 4 weeks post-inoculation (Fig. 4A-B). PEDV-infected pig serum can recognize the *E.coli*-expressed nsp10 protein, indicating that it has good immunogenicity (Fig. 4C). Compared with commercial PEDV ELISA kit, nsp10-ELISA had high degree of consistency to detect sera samples of PEDV-infected or uninfected pig (Fig. 4D). It is suggested that PEDV nsp10 may serve as the candidate for developing PEDV detection kit.

PEDV is the main causative pathogen of viral diarrhea in piglets which could cause rapid destruction and detachment of the intestinal physical structure(Wang et al., 2018). Over the years, a number of works has been done to investigate PEDV pathogenesis and prevention. Vaccination against PEDV is an important measure to prevent and control the rate of PEDV infection(Chen et al., 2017). However, due to the continued mutation of PEDV genome (Lin et al., 2016), the current available vaccines cannot provide complete protection for pigs. Therefore, it is important that PEDV infection can be efficiently detected when outbreaks of disease occur or effective vaccine will be developed. Whole virus vaccines are mainly traditional vaccines of PEDV such as live attenuated vaccines and inactivated vaccines. For live attenuated vaccines, the risk of reversion to high virulence strain limits its applicability, although live attenuated vaccines possess highly immunogenic (Lauring et al., 2010; Yong et al., 2019). As to inactivated vaccines, there are several advantages including good safety profile and ease production. However, incomplete inactivation and multiple immunizations are some disadvantages that cannot be neglected(DeZure et al., 2016). Compared with whole virus vaccines, subunit vaccines represent promising approaches because of high safety, no contagious viral nucleic acids, uniform antigen(Du et al., 2016; Yap and Smith, 2010). The S protein of PEDV is main target used for developing subunit vaccines since it can induce neutralizing antibodies and interfere virus entry into host cells(Makadiya et al., 2016). Non-structural protein of coronavirus does not provide protection as neutralization antibodies against virus infection. However, antibodies against non-structural protein also can provide protective immunity by Fcγ receptor-mediated viral clearance, complement-mediated cytotoxicity and complement-independent phagocytosis(Rastogi et al., 2016; Reyes-Sandoval and Ludert, 2019; Wan et al., 2021). In addition, non-structural protein can trigger cellular immune response that protects against virus infection(Grubor-Bauk et al., 2019; Mishra et al., 2020). Subsequently, accumulating data have emerged as to develop non-structural protein-based vaccines(Bailey et al., 2018; Lin et al., 1998). In addition to non-structural protein-based vaccines, non-structural protein-specific antibodies would also be expected to be used for developing antiviral drugs that inhibit virus replication. Research reported that PRRSV nsp9-specific nanobodies that delivered into MARC-145 cells and PAMs can efficiently inhibited the replication of several PRRSV strains(Liu et al., 2015; Wang et al., 2019). In this study, the results showed that expression level of cytokine IL-2, IL-4, IL-10, TNF-α, IFN-γ in splenocytes or sera from immunized mice increased, indicating that nsp10 can promote cellular immunity (Fig. 5B-C). Modified seroneutralization test indicated that nsp10 antibodies from immunized mouse sera may protect cells from PEDV infection (Fig. 6). In addition, we aware of several limitations of this study, which all experiments involving the immunogenicity of PEDV nsp10 were carried out in mice. As pigs are the natural host of PEDV, in vivo studies on the protective immunity of PEDV nsp10 are certainly required in the future.

In conclusion, full-length nsp10 amplified from CV777 strain was expressed in *E.coli*, which protein yields were considerable high. Immunization challenge studies in mice, demonstrate that PEDV nsp10 induced robustly humoral and cellular immune responses. Furthermore, nsp10 antibodies from immunized mouse sera may have protective effect on cells from PEDV infection. Despite these potential limitations, we believe that the nsp10 protein has potential for use in developing effective and safe anti-PEDV drugs for PED prevention.

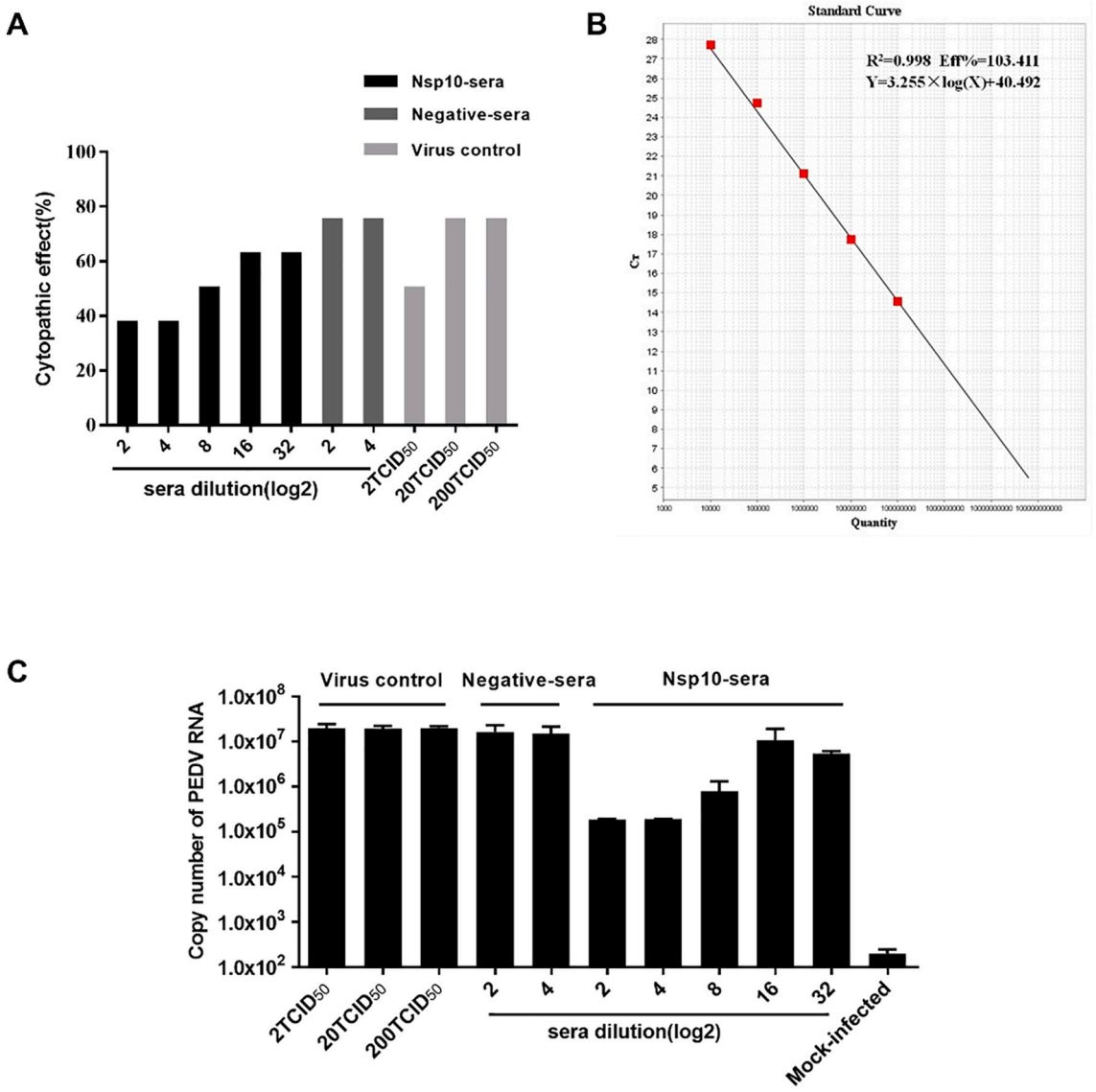


Fig. 6. Mice serological protection assay and absolute PEDV quantitation.
(A) The protective effect of mice sera at different concentrations. The mice sera were serially diluted, and the protective effects on PEDV infection at cell level were determined by observing CPE. (B) Standard curve and equation of PEDV N gene. Tenfold serial dilution ranging from 10^2 to 10^7 copies of pCAGGS-HA-N plasmid were tested in Real-time PCR. A standard curve graph is made by plotting the C_T values on the x axis. (C) Quantiative RT-PCR was performed to detect PEDV N expression in serum-treated cells and PEDV-infection cells.

Declaration of Competing Interest

There were no potential conflicts (financial, professional or personal) that are relevant to the manuscript.

Acknowledgements

This work was supported by 31860038 and 31960699 from the National Natural Science Foundation of China.

References

Anand, K., Palm, G.J., Mesters, J.R., Siddell, S.G., Ziebuhr, J., Hilgenfeld, R., 2002. Structure of coronavirus main proteinase reveals combination of a chymotrypsin fold with an extra alpha-helical domain. EMBO J. 21, 3213–3224.

Bailey, M.J., Duehr, J., Dulin, H., Broecker, F., Brown, J.A., Arumemi, F.O., Bermudez Gonzalez, M.C., Leyva-Grado, V.H., Evans, M.J., Simon, V., Lim, J.K., Krammer, F., Hai, R., Palese, P., Tan, G.S., 2018. Human antibodies targeting Zika virus NS1 provide protection against disease in a mouse model. Nat. Commun. 9, 4560.

Benova, K., Hanckova, M., Koci, K., Kudelova, M., Betakova, T., 2020. T cells and their function in the immune response to viruses. Acta Virol. 64, 131–143.

Bouvet, M., Debarnot, C., Imbert, I., Selisko, B., Snijder, E.J., Canard, B., Decroly, E., 2010. In vitro reconstitution of SARS-coronavirus mRNA cap methylation. PLoS Pathog. 6, e1000863.

Bouvet, M., Imbert, I., Subissi, L., Gluais, L., Canard, B., Decroly, E., 2012. RNA 3′-end mismatch excision by the severe acute respiratory syndrome coronavirus nonstructural protein nsp10/nsp14 exoribonuclease complex. Proc. Natl. Acad. Sci. U. S. A. 109, 9372–9377.

Bouvet, M., Lugari, A., Posthuma, C.C., Zevenhoven, J.C., Bernard, S., Betzi, S., Imbert, I., Canard, B., Guillemot, J.C., Lecine, P., Pfefferle, S., Drosten, C., Snijder, E.J., Decroly, E., Morelli, X., 2014. Coronavirus Nsp10, a critical co-factor for activation of multiple replicative enzymes. J. Biol. Chem. 289, 25783–25796.

Chen, N., Li, S., Zhou, R., Zhu, M., He, S., Ye, M., Huang, Y., Li, S., Zhu, C., Xia, P., Zhu, J., 2017. Two novel porcine epidemic diarrhea virus (PEDV) recombinants from a natural recombinant and distinct subtypes of PEDV variants. Virus Res. 242, 90–95.

Decroly, E., Debarnot, C., Ferron, F., Bouvet, M., Coutard, B., Imbert, I., Gluais, L., Papageorgiou, N., Sharff, A., Bricogne, G., Ortiz-Lombardia, M., Lescar, J., Canard, B., 2011. Crystal structure and functional analysis of the SARS-coronavirus RNA cap 2′-O-methyltransferase nsp10/nsp16 complex. PLoS Pathog. 7, e1002059.

DeZure, A.D., Berkowitz, N.M., Graham, B.S., Ledgerwood, J.E., 2016. Whole-inactivated and virus-like particle vaccine strategies for chikungunya virus. J. Infect. Dis. 214, S497–S499.

Donaldson, E.F., Graham, R.L., Sims, A.C., Denison, M.R., Baric, R.S., 2007a. Analysis of murine hepatitis virus strain A59 temperature-sensitive mutant TS-LA6 suggests that nsp10 plays a critical role in polyprotein processing. J. Virol. 81, 7086–7098.
Donaldson, E.F., Sims, A.C., Graham, R.L., Denison, M.R., Baric, R.S., 2007b. Murine hepatitis virus replicase protein nsp10 is a critical regulator of viral RNA synthesis. J. Virol. 81, 6356–6368.
Du, L., Tai, W., Zhou, Y., Jiang, S., 2016. Vaccines for the prevention against the threat of MERS-CoV. Exp. Rev. Vaccin. 15, 1123–1134.
Duarte, M., Gelfi, J., Lambert, P., Rasschaert, D., Laude, H., 1993. Genome organization of porcine epidemic diarrhoea virus. Adv. Exp. Med. Biol. 342, 55–60.
Gauger, P.C., Vincent, A.L., 2014. Serum virus neutralization assay for detection and quantitation of serum-neutralizing antibodies to influenza A virus in swine. Methods Mol. Biol. 1161, 313–324.
Grubor-Bauk, B., Wijesundara, D.K., Masavuli, M., Abbink, P., Peterson, R.L., Prow, N.A., Larocca, R.A., Mekonnen, Z.A., Shrestha, A., Eyre, N.S., Beard, M.R., Gummow, J., Carr, J., Robertson, S.A., Hayball, J.D., Barouch, D.H., Gowans, E.J., 2019. NS1 DNA vaccination protects against Zika infection through T cell-mediated immunity in immunocompetent mice. Sci. Adv. 5, eaax2388.
Joseph, J.S., Saikatendu, K.S., Subramanian, V., Neuman, B.W., Brooun, A., Griffith, M., Moy, K., Yadav, M.K., Velasquez, J., Buchmeier, M.J., Stevens, R.C., Kuhn, P., 2006. Crystal structure of nonstructural protein 10 from the severe acute respiratory syndrome coronavirus reveals a novel fold with two zinc-binding motifs. J. Virol. 80, 7894–7901.
Ke, M., Chen, Y., Wu, A., Sun, Y., Su, C., Wu, H., Jin, X., Tao, J., Wang, Y., Ma, X., Pan, J. A., Guo, D., 2012. Short peptides derived from the interaction domain of SARS coronavirus nonstructural protein nsp10 can suppress the 2'-O-methyltransferase activity of nsp10/nsp16 complex. Virus Res. 167, 322–328.
Lauring, A.S., Jones, J.O., Andino, R., 2010. Rationalizing the development of live attenuated virus vaccines. Nat. Biotechnol. 28, 573–579.
Li, W., Li, H., Liu, Y., Pan, Y., Deng, F., Song, Y., Tang, X., He, Q., 2012. New variants of porcine epidemic diarrhea virus, China, 2011. Emerg. Infect. Dis. 18, 1350–1353.
Li, S., Zhao, H., Yang, H., Hou, W., Cruz-Cosme, R., Cao, R., Chen, C., Wang, W., Xu, L., Zhang, J., Zhong, W., Xia, N., Tang, Q., Cheng, T., 2020. Rapid neutralization testing system for Zika virus based on an enzyme-linked Immunospot assay. ACS Infect Dis 6, 811–819.
Lin, Y.L., Chen, L.K., Liao, C.L., Yeh, C.T., Ma, S.H., Chen, J.L., Huang, Y.L., Chen, S.S., Chiang, H.Y., 1998. DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. J. Virol. 72, 191–200.
Lin, H., Chen, L., Gao, L., Yuan, X., Ma, Z., Fan, H., 2016. Epidemic strain YC2014 of porcine epidemic diarrhea virus could provide piglets against homologous challenge. Virol. J. 13, 68.
Lin, S., Chen, H., Ye, F., Chen, Z., Yang, F., Zheng, Y., Cao, Y., Qiao, J., Yang, S., Lu, G., 2020. Crystal structure of SARS-CoV-2 nsp10/nsp16 2'-O-methylase and its implication on antiviral drug design. Sign. Transd. Targ. Ther. 5, 131.
Liu, H., Wang, Y., Duan, H., Zhang, A., Liang, C., Gao, J., Zhang, C., Huang, B., Li, Q., Li, N., Xiao, S., Zhou, E.M., 2015. An intracellularly expressed Nsp9-specific nanobody in MARC-145 cells inhibits porcine reproductive and respiratory syndrome virus replication. Vet. Microbiol. 181, 252–260.
Lu, Y., Cai, H., Lu, M., Ma, Y., Li, A., Gao, Y., Zhou, J., Gu, H., Li, J., Gu, J., 2020. Porcine epidemic diarrhea virus deficient in RNA cap guanine-N-7 methylation is attenuated and induces higher type I and III interferon responses. J. Virol. 94.
Makadiya, N., Brownlie, R., van den Hurk, J., Berube, N., Allan, B., Gerdts, V., Zakhartchouk, A., 2016. S1 domain of the porcine epidemic diarrhea virus spike protein as a vaccine antigen. Virol. J. 13, 57.
Matsuda, K., 2017. PCR-based detection methods for single-nucleotide polymorphism or mutation: real-time PCR and its substantial contribution toward technological refinement. Adv. Clin. Chem. 80, 45–72.
Matthes, N., Mesters, J.R., Coutard, B., Canard, B., Snijder, E.J., Moll, R., Hilgenfeld, R., 2006. The non-structural protein Nsp10 of mouse hepatitis virus binds zinc ions and nucleic acids. FEBS Lett. 580, 4143–4149.
Mishra, N., Boudewijns, R., Schmid, M.A., Marques, R.E., Sharma, S., Neyts, J., Dallmeier, K., 2020. A chimeric Japanese encephalitis vaccine protects against lethal yellow fever virus infection without inducing neutralizing antibodies. mBio 11.
Pensaert, M.B., Martelli, P., 2016. Porcine epidemic diarrhea: a retrospect from Europe and matters of debate. Virus Res. 226, 1–6.
Rastogi, M., Sharma, N., Singh, S.K., 2016. Flavivirus NS1: a multifaceted enigmatic viral protein. Virol. J. 13, 131.
Reyes-Sandoval, A., Ludert, J.E., 2019. The dual role of the antibody response against the flavivirus non-structural protein 1 (NS1) in protection and immuno-pathogenesis. Front. Immunol. 10, 1651.
Shi, P., Su, Y., Li, R., Liang, Z., Dong, S., Huang, J., 2019. PEDV nsp16 negatively regulates innate immunity to promote viral proliferation. Virus Res. 265, 57–66.
Snijder, E.J., Decroly, E., Ziebuhr, J., 2016. The nonstructural proteins directing coronavirus RNA synthesis and processing. Adv. Virus Res. 96, 59–126.
Song, D., Park, B., 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. Virus Genes 44, 167–175.
Su, D., Lou, Z., Sun, F., Zhai, Y., Yang, H., Zhang, R., Joachimiak, A., Zhang, X.C., Bartlam, M., Rao, Z., 2006. Dodecamer structure of severe acute respiratory syndrome coronavirus nonstructural protein nsp10. J. Virol. 80, 7902–7908.
Subissi, L., Imbert, I., Ferron, F., Collet, A., Coutard, B., Decroly, E., Canard, B., 2014. SARS-CoV ORF1b-encoded nonstructural proteins 12-16: replicative enzymes as antiviral targets. Antivir. Res. 101, 122–130.
Sun, R.Q., Cai, R.J., Chen, Y.Q., Liang, P.S., Chen, D.K., Song, C.X., 2012. Outbreak of porcine epidemic diarrhea in suckling piglets, China. Emerg. Infect. Dis. 18, 161–163.
Tian, P.F., Jin, Y.L., Xing, G., Qv, L.L., Huang, Y.W., Zhou, J.Y., 2014. Evidence of recombinant strains of porcine epidemic diarrhea virus, United States, 2013. Emerg. Infect. Dis. 20, 1735–1738.
Wan, J., Wang, T., Xu, J., Ouyang, T., Wang, Q., Zhang, Y., Weng, S., Li, Y., Wang, Y., Xin, X., Wang, X., Li, S., Kong, L., 2021. Novel Japanese encephalitis virus NS1-based vaccine: truncated NS1 fused with *E. coli* heat labile enterotoxin B subunit. EBioMedicine 67, 103353.
Wang, B., Hua, R.H., Tian, Z.J., Chen, N.S., Zhao, F.R., Liu, T.Q., Wang, Y.F., Tong, G.Z., 2009. Identification of a virus-specific and conserved B-cell epitope on NS1 protein of Japanese encephalitis virus. Virus Res. 141, 90–95.
Wang, Y., Sun, Y., Wu, A., Xu, S., Pan, R., Zeng, C., Jin, X., Ge, X., Shi, Z., Ahola, T., Chen, Y., Guo, D., 2015. Coronavirus nsp10/nsp16 methyltransferase can be targeted by nsp10-derived peptide in vitro and in vivo to reduce replication and pathogenesis. J. Virol. 89, 8416–8427.
Wang, D., Fang, L., Xiao, S., 2016. Porcine epidemic diarrhea in China. Virus Res. 226, 7–13.
Wang, X.Y., Ji, C.J., Zhang, X., Xu, D.P., Zhang, D.L., 2018. Infection, genetic and virulence characteristics of porcine epidemic diarrhea virus in Northwest China. Infect. Genet. Evol. 62, 34–39.
Wang, L., Zhang, L., Huang, B., Li, K., Hou, G., Zhao, Q., Wu, C., Nan, Y., Du, T., Mu, Y., Lan, J., Chen, H., Zhou, E.M., 2019. A nanobody targeting viral nonstructural protein 9 inhibits porcine reproductive and respiratory syndrome virus replication. J. Virol. 93.
Yap, Y.K., Smith, D.R., 2010. Strategies for the plant-based expression of dengue subunit vaccines. Biotechnol. Appl. Biochem. 57, 47–53.
Yong, C.Y., Ong, H.K., Yeap, S.K., Ho, K.L., Tan, W.S., 2019. Recent advances in the vaccine development against Middle East respiratory syndrome-coronavirus. Front. Microbiol. 10, 1781.