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# Identification of a peptide derived from the heptad repeat 2 region of the porcine epidemic diarrhea virus (PEDV) spike glycoprotein that is capable of suppressing PEDV entry and inducing neutralizing antibodies

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## ABSTRACT

Heptad repeat (HR) regions are highly conserved motifs located in the glycoproteins of enveloped viruses that form a six-helix bundle structure and is important in the process of virus fusion. Peptides derived from the HR regions of some viruses have also been shown to inhibit viral entry. Porcine epidemic diarrhea virus (PEDV) was predicted to have HR regions (HR1 and HR2) in the spike glycoprotein S2 subunit. Based on this analysis, six peptides derived from HR1 and HR2 were selected, expressed in *Escherichia coli*, purified, and characterized. Three peptides (HR2M, HR2L and HR2P) were identified as potential competitive inhibitors in PEDV *in vitro* infection assays, with the HR2P peptide representing the most potent inhibitor. Further study indicated that immunization of HR2P in mice elicited antibodies capable of neutralizing PEDV infection *in vitro*. These results demonstrate that the HR2P peptide and anti-HR2P antibody can serve as a tool for dissecting the fusion mechanism of PEDV, guiding the search for potent inhibitors with therapeutic value against PEDV infection.

## 1. Introduction

Porcine epidemic diarrhea virus (PEDV; family *Coronaviridae*, order *Nidovirales*) is an enveloped, positive-sense, single-stranded RNA virus which readily infects pigs, resulting in a serious and highly contagious swine disease, porcine epidemic diarrhea (PED), characterized by severe diarrhea and dehydration in suckling piglets (Lee, 2015; Lin et al., 2014; Puranaveja et al., 2009; Wang et al., 2013). PED was first identified in Europe in the early 1970s, and the virus was first isolated in Belgium in 1978 (Pensaert and de Bouck, 1978). PEDV outbreaks have since been reported in many swine-producing regions including China (Chen et al., 2011; Zhou et al., 2012), the USA (Huang et al., 2013), Canada (Ojkic et al., 2015) and Europe (Dastjerdi et al., 2015; Mesquita et al., 2015). Although most sow herds had previously been vaccinated with a CV777-based inactivated vaccine, a large-scale outbreak of PED was observed in suckling piglets on many farms in China starting in late 2010, which has resulted in increased economic losses associated with high morbidity and mortality. On the basis of phylogenetic differences of full-length genomic sequences, PEDV strains were classified into two

distinct genogroups, genogroup-1 (G1) and genogroup-2 (G2). PEDV field strains isolated before 2010 including CV777 and the derived vaccine strains belonged to G1, whereas the emergent PEDV strains isolated since 2011 in China and US fell into G2 (Huang et al., 2013). The ongoing importance of this disease necessitates the development of an effective vaccine and antiviral treatment.

Coronaviruses have the largest genome among RNA viruses, which encodes several proteins that are embedded on the surface of the viral envelope. It is generally believed that the spike (S) glycoprotein initially binds to the cell surface receptor, causing a conformational change that exposes the fusion peptide to the target cell membrane. Subsequently, highly conserved heptad repeat (HR) regions interact to form six-helix bundle (6-HB) structures, which contribute to the fusion of virus and cell membranes (Eckert and Kim, 2001). The S glycoprotein consists of S1 and S2 subunits; S1 is located at the N-terminus and binds to the cell receptor (Ballesteros et al., 1997), whereas S2 is involved in the virus-cell fusion process. Many *in vitro* studies of coronavirus infection have reported that exogenous soluble HR2 peptide can bind to viral HR1, thereby efficiently blocking viral entry into the cell and inhibiting

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replication (Bosch et al., 2004; Deng et al., 2006; Liu et al., 2004, 2009). Based on these findings, entry inhibitors have also been identified for coronaviruses and other viruses, such as HR2 for Severe Acute Respiratory Syndrome coronaviruses (SARS-CoV; (Yuan et al., 2004), Middle East Respiratory Syndrome coronavirus (MERS-CoV; (Gao et al., 2013; Lu et al., 2014; Zhao et al., 2013), mouse hepatitis virus (MHV; (Bosch et al., 2003), T20 from human immunodeficiency virus (HIV)-1 (Imai et al., 2000), and GP610 for Ebola virus (Watanabe et al., 2000).

As a typical class I enveloped virus, PEDV is presumed to use a similar membrane fusion mechanism for viral entry. Analysis of the PEDV S2 subunit predicted the presence of two HRs similar to other enveloped viruses (Liu et al., 2013; Ma et al., 2005; Zheng et al., 2006). Based on the study on the fusion mechanism of coronaviruses described above, a general approach had been developed that was successfully used to study antiviral peptides in other coronaviruses such as SARS-CoV, MHV and MERS-CoV. Similarly, in this study, in order to find a potent inhibitor of viral entry, a series of peptides overlapping the predicted HR regions in the PEDV S2 subunit were designed and screened for inhibitory effects in a G2 PEDV infection assay. Cross-reactivity of these peptides with G1 PEDV was also investigated. Three peptides (HR2M, HR2L and HR2P) that were able to inhibit entry were identified, with HR2P being the most potent. Furthermore, immunization of HR2P in mice elicited antibodies that were capable of neutralizing PEDV infection *in vitro*.

## 2. Materials and methods

### 2.1. Cell cultures

Vero (ATCC CCL-81) or Huh7 (human hepatocellular carcinoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (w/v) in a humidified 5% CO<sub>2</sub> incubator at 37 °C.

### 2.2. Generation of PEDV virus stocks

We used two PEDV strains from different genogroups in this study. The virus stock of a recombinant G2 PEDV (strain ZJU/G2/2013, GenBank Accession No. KU558701; Qin et al., 2017) expressing green fluorescent protein (GFP), designated as PEDV-GFP in this study, was generated by transfection of Vero cells with a PEDV infectious cDNA clone (Huang YW et al., unpublished data) followed by passaging in Vero cells. The PEDV G1 strain ZJU/G1/2013 (GenBank Accession No. KU664503) was propagated in Vero cells. Briefly, Vero cells were seeded at 70% confluency in 6-well plates and incubated overnight. After washing the cells with PBS, viruses at MOI = 0.1 diluted in maintenance medium with trypsin (MMT) containing DMEM, 0.3% tryptose phosphate broth (TPB), 5 µg/mL trypsin (Sigma, Cat#T7186-50TAB, St Louis, MO, USA), and 1% penicillin/streptomycin, were added to each well. After 2 h incubation at 37 °C, the cells were washed with PBS and then cultured with MMT. Cells were observed daily to check for any cytopathic effect (CPE), particularly syncytium formation. When CPE was obvious, whole cell cultures (supernatant and cells) were harvested and subjected to three freeze-thaw cycles prior to removal of cell debris by centrifugation. The virus titers of PEDV-GFP and PEDV-G1 were determined by endpoint dilutions as 50% tissue culture infective dose (TCID<sub>50</sub>) on Vero cells, respectively. Virus stocks were stored at –80 °C until use.

### 2.3. Prediction and analysis of the heptad repeat regions of PEDV

HR1 and HR2 regions of the S protein PEDV of the strain ZJU/G2/2013 were predicted using the computer software LearnCoil-VMF (<http://night-ingle.lcs.mit.edu/cgi-bin/vmf>) (Singh et al., 1999). Hydrophathy plots corresponding to the HR motifs of PEDV were obtained

**Table 1**

Sequence of the oligonucleotides used for PCR.

Primer Name	Sequence (5'-3') <sup>a</sup>
HR1-F	GTCGGATCCCTCAATTATCTTGCTC
HR1-R	CCGCTCGAGTCACTCATTAACTTTTGTCTGTGC
HR1M-F	GTTGGATCCCGGAACCAGCAATTG
HR1M-R	ATACTCGAGTCATTGACGCTGTACGGT
HR1L-F	GTTGGATCCTCTGCTATTGGTAAT
HR1L-R	ATACTCGAGTCATTGGAAGTTGTGTTG
HR2-F	GTTGGATCCAAGCTCACTGGTGA
HR2-R	ATACTCGAGTCAGAGCCACTCAAGGT
HR2M-F	GTGGATCCAATGCCACTTATCTTA
HR2M-R	ATACTCGAGTCAAAGGTCAAAGTGTG
HR2L-F	GTGGATCCCTTTAGATGTTTTTA
HR2L-R	ATACTCGAGTCATGTCTCAACTCGGT
HR2P-F	GTGGATCCAATGCCACTTATCTTA
HR2P-R	ATACTCGAGTCACTCAACTCGGTTGAG

<sup>a</sup> Restriction endonuclease sites: *Bam*HI (italic) and *Xho*I (underlined).

using Tmpred program (ExpASY, Swiss Institute of Bioinformatics, <http://www.ch.embnet.org>). To optimize solubility of expressed proteins, the HR1 and HR2 regions of PEDV were truncated or extended *in silico*, respectively, to design six peptides, on the basis of the multiple sequence alignments with different coronaviruses.

### 2.4. Construction of recombinant plasmids

Six DNA fragments corresponding to the desired peptides were amplified through polymerase chain reaction (PCR) on full-length PEDV-GFP S constructs. Two unique restriction sites, *Bam*HI or *Xho*I, were introduced at the upstream primers or the downstream primers for all DNA fragments, respectively. Amplicons were digested with *Bam*HI/*Xho*I and ligated into an appropriately digested pGEX-4T-1 vector containing a Glutathione S-transferase (GST) tag. The corresponding primer pairs are listed in Table 1.

### 2.5. Expression and purification of recombinant GST-fusion peptides

Recombinant clones were transformed into competent *E. coli* BL21 (DE3), and a single colony was inoculated into Luria-Bertani (LB) broth containing 50 mg/L ampicillin (Sigma, USA) and incubated at 37 °C. Overnight cultures were transferred into 100 mL of fresh LB medium for large-scale protein production at 37 °C. When the culture optical density (OD<sub>590</sub>) reached 0.4, peptide synthesis was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma, USA) and grown for another 8 h at 30 °C until cell harvest. Recombinant GST-fused peptides were purified with ProteinIso GST Resin (Tansbionovo, Beijing, China), according to the manufacturer's instructions with some minor modifications. Briefly, cells were harvested by centrifugation at 8000 × g for 10 min at 4 °C and the pellet was resuspended in 30 mL of ice-cold PBS (pH = 7.4). After sonication, the suspensions were centrifuged at 12,000 × g for 15 min at 4 °C and supernatants filtered through a 0.45 µm membrane before being bound to ProteinIso GST Resin. After loading, columns were washed with 10 column volumes of PBS and GST-fusion peptides were eluted in 20 mM reduced glutathione (Sigma, USA). Eluted proteins were dialyzed against PBS and adjusted to the desired concentration by ultrafiltration through 10 K membranes (Millipore). Purified, soluble GST-fusion peptides were analyzed by SDS-PAGE and stored at –80 °C until further analysis. Protein concentration was measured by using the enhanced BAC protein assay kit (Beyotime, China) according to the manufacturer's instruction.

### 2.6. Cytotoxicity assays

The cytotoxicity of peptides in Huh7 cells was determined using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, China),

following the manufacturer's instructions. Briefly, Huh-7 cells were seeded in 96-well plates at a density of  $1 \times 10^5$ /well and maintained at 37 °C in 5% CO<sub>2</sub> for 24 h. Cells were treated with peptides (40 μM) for 24, 48 or 72 h, and 20 μL of MTT solution (5 mg/mL, in PBS) was added to each well. After incubation for 4 h at 37 °C, 100 μL of Formazan solution was added, mixed, and incubated 2 h at 37 °C. The optical density of each well was measured at 570 nm.

## 2.7. Screening of peptides for effective inhibition of PEDV infection

Dose-dependence of inhibitory activity was determined as described previously with a few modifications (Chi et al., 2013; Wang et al., 2011). Huh7 cells were seeded at 70% confluency in 48-well plates, and infected 24 h later with PEDV-GFP (MOI = 0.01) mixed with recombinant HR peptides at a range of concentrations (5, 10, 20, 40 μM) for 2 h at 37 °C. Uninfected control cultures were also treated with soluble peptides diluted in maintenance media (MM) containing DMEM and 0.3% tryptose phosphate broth (TPB) at concentrations of 5, 10, 20, and 40 μM. Following treatment, the virus-peptide mixtures were removed, MM was added and incubated for 48 h at 37 °C, at which point the number of GFP positive cell clusters was counted (quantified as fluorescent focus-forming unit [FFU]). Inhibitory concentration 50% (IC<sub>50</sub>) values were calculated according to previously described (Lu et al., 2014). Results are expressed as the average of triplicates ± standard deviation. Analysis of variance (ANOVA) was used for assessment of the inhibitory percentage of PEDV infection of each GST fusion peptide in comparison with the GST control protein at different doses. Statistical significance was set to a P-value of 0.05.

To determine the effect of timing on inhibitory activity, three different methods of cell monolayer treatment with peptides at a dose of 40 μM were used (Chi et al., 2014; Lopper and Compton, 2004): 1) Cell pre-treatment, in which cells were incubated with peptides for 30 min at 4 °C, peptides removed, cells washed with PBS and infected with PEDV-GFP (MOI = 0.01) for 1 h at 37 °C; 2) Co-treatment, where cells were incubated with peptides in the presence of viral inoculum (MOI = 0.01) for 1 h at 37 °C; and 3) post-treatment, in which cells were infected with virus (MOI = 0.01) for 45 min at 37 °C, washed with PBS, and then treated with peptides for 1 h at 37 °C. In all cases, cell monolayers were subsequently incubated in MM for 2 d at 37 °C, fixed in 4% paraformaldehyde, and was assessed by counting GFP-positive cell clusters. Results are expressed as the average of triplicates ± standard deviation and all experiments were conducted in parallel with each GST fusion peptide and the GST control in different treatment.

## 2.8. Generation of polyclonal antiserum against the PEDV HR2P peptide

The PEDV HR2P peptide was cloned into the prokaryotic expression vector pET32a containing a His tag, and recombinant protein was expressed and purified with a His-fusion purification column (Novoprotein, Shanghai, China), as described previously (Huang et al., 2011). The purified soluble peptide was used as an immunogen (50 μg of each mouse) for the generation of polyclonal antibodies in mice, with booster immunizations of 100 μg per mouse administered at 14, 24 and 34 days post-vaccination. Seven days after the final booster, serum was collected and used to detect HR2P by means of ELISA assay.

## 2.9. Enzyme-linked immunosorbent assay (ELISA)

The purified soluble PEDV HR2P-GST was diluted in buffer (50 mM Na<sub>2</sub>CO<sub>3</sub>, 50 mM NaHCO<sub>3</sub>, pH 9.6) to a final concentration of 10 μg/mL, and 100 μL was used to coat each well of a 96-well plate at 37 °C for 1 h. Plates were washed three times with PBS with 0.05% Tween (PBST), followed by blocking in 5% bovine serum albumin at 37 °C for 1 h. After three washes with PBST, plates were incubated with polyclonal anti-HR2P (2-fold serial dilutions) at 37 °C for 1 h. After three washes with PBST, an HRP-conjugated goat anti-mouse IgG antibody (1:2000

dilution) was added at 37 °C for 1 h. After three washes with PBST, 1 × TMB (Tetramethyl Benzidine) substrate (Invitrogen, CA, USA) was added and incubated at 37 °C for 15 min, then stopped by addition of 2 M H<sub>2</sub>SO<sub>4</sub>. The absorbance at 450 nm was quantified by a microplate reader (Infinite® 200 Pro NanoQuant, Tecan, Switzerland); sample-to-negative OD<sub>450</sub> ratios greater than 2.1 were considered significantly positive (Chow et al., 1992).

## 2.10. Virus neutralizing (VN) assay

The VN assay was performed on monolayers of Huh7 cells, according to previous reports (Fan et al., 2013). Briefly, serial dilutions of 50 μL of pre-immunized serum, anti-HR2P antiserum or an anti-PEDV-S2 monoclonal antibody (a gift from Dr. Ying Fang, Kansas State University) were each mixed with 100 FFU of PEDV-GFP in MM, incubated at 37 °C for 1 h to form virus-antibody complexes, and added to Huh7 cells grown in 48-well plates with DMEM containing 10% FBS. After 1 h incubation at 37 °C, unabsorbed viruses were thoroughly removed by three PBS washes. Cells were fixed in 4% paraformaldehyde at 36 h post-infection, and GFP-positive cells were counted as FFU by immunofluorescence microscopy. The number of FFU was quantified and normalized to the number derived from control wells in which virus was mixed with pre-immune sera.

## 3. Results

### 3.1. Organization of the HR regions in the PEDV S glycoprotein

The HR regions of the PEDV (strain ZJU/G2/2013 or PEDV-GFP) S glycoprotein were identified based on predictions by the LearnCoil-VMF program (Fig. 1A) and were further verified by a structural-based multiple amino acid sequence alignment of two PEDV strains representing G1 (ZJU/G1/2013) and G2 (PEDV-GFP) with several other coronaviruses: human coronavirus (HCoV) NL63, transmissible gastroenteritis virus (TGEV), MERS-CoV, SARS-CoV, feline infectious peritonitis virus (FIPV), HCoV-OC43, HCoV-229E, porcine deltacoronavirus (PDCoV), avian infectious bronchitis virus (IBV), porcine respiratory coronavirus (PRCoV) and MHV (Fig. 1B–C). The S2 subunit of PEDV, like SARS-CoV and HCoV-NL63, contains two HR domains, the HR1 region (residues 982–1121) and the HR2 region (residues 1278–1317). A fusion peptide (FP) in the upstream of HR1, as well as a transmembrane domain (TM) and a cytoplasmic tail (CT) in the downstream of HR2, were also identified (Fig. 1A). Similar to the determined HR structures of HCoV-NL63, SARS-CoV and MERS-CoV (Zheng et al., 2006; Liu et al., 2004; Lu et al., 2014), we hypothesized that, during the fusion of PEDV and cell membrane, the S2 subunit of PEDV changes conformation by forming a 6-HB fusion core between HR1 and HR2 and inserting the FP into the target cell membrane. However, attempts to determine the crystal structure of the PEDV-S2 6-HB fusion core did not succeed (data not shown).

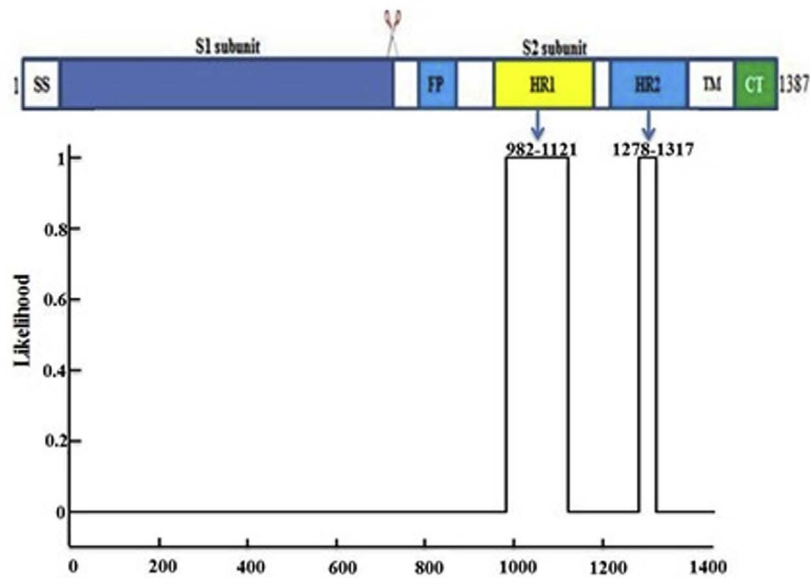
### 3.2. Recombinant GST-fusion peptides from the HR regions were soluble and non-cytotoxic

To empirically identify the HR regions and to screen the peptide inhibitors within the S2 subunit of PEDV, six peptides, designated as HR1M, HR1L, HR2, HR2M, HR2L and HR2P corresponding to the HR1 and HR2 regions were designed (Fig. 2A) and expressed as GST fusion proteins in *E. coli*. The single GST proteins expressed by the expression vector pGEX-4T-1 served as the control. The products were soluble, and were subsequently purified and analyzed by 10% SDS-PAGE, respectively, yielding bands of expected size (Fig. 2B). The expression levels of these products varied, with the weakness amounts shown in HR1M, HR1L (an additional unknown band appeared), and HR2 peptides, likely attributed to the distinct hydrophobic features of these peptides.

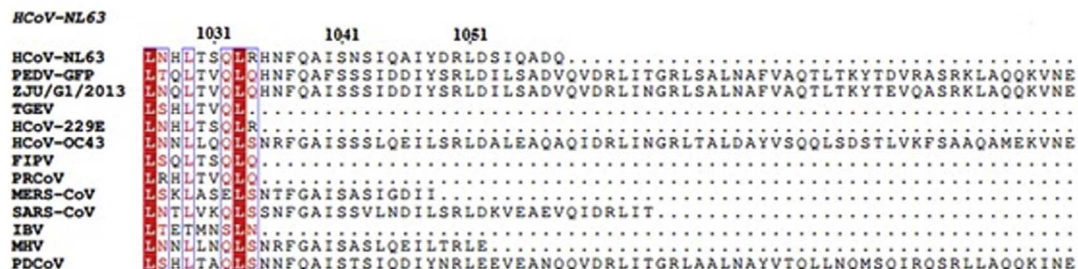
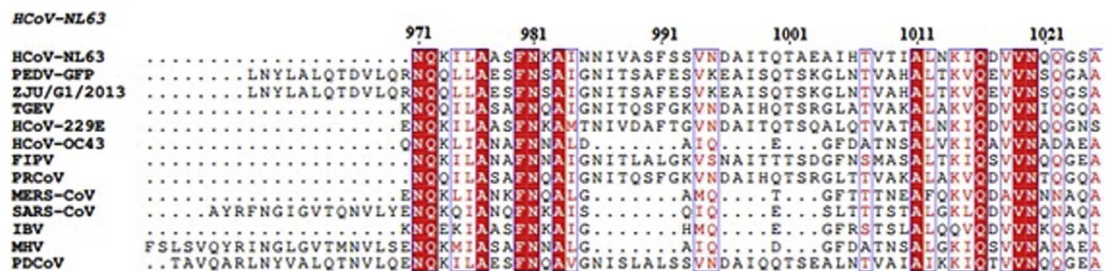
Since PEDV can productively infect Huh7 cells without addition of



A



B



C

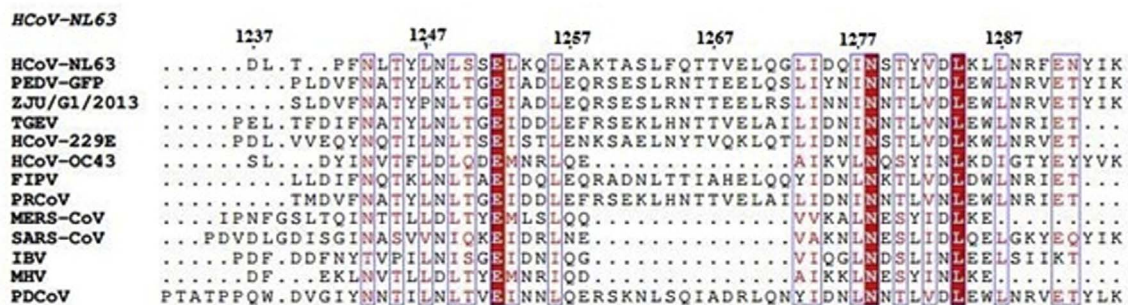


Fig. 1. Prediction of the HR regions of the PEDV S glycoprotein (A) Upper panel: A schematic of the S glycoprotein; S1/S2 cleavage site indicated by a scissor, SS (signal peptide); HR1 and HR2 (heptad repeat regions); TM (transmembrane domain); CT (cytoplasmic tail domain). Lower panel: HR1 and HR2 were predicted by the LearnCoil-VMF program. Multiple sequence alignment (ClustalX1.8 and ESPrpt3.1 programs) of (B) the HR1 and (C) HR2 regions; sequences shown for HCoV-NL63 (Human coronavirus NL63; GenBank accession no. AAS58177), ZJU/G1/2013 (AOC38021), TGEV (transmissible gastroenteritis virus; AAB30949), HCoV-229E (Human coronavirus 229E; AAK32191), PRCoV (porcine respiratory coronavirus; CAA42686), FIPV (feline infectious peritonitis virus; BAA06805), MHV (mouse hepatitis virus; P11225), HCoV-OC43 (S44241), MERS-CoV (AHC74098), SARS-CoV (AAP13441), IBV (avian infectious bronchitis virus; AAO34396), PDCoV (porcine deltacoronavirus; ANI85846). Residues highlighted in red are completely conserved. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

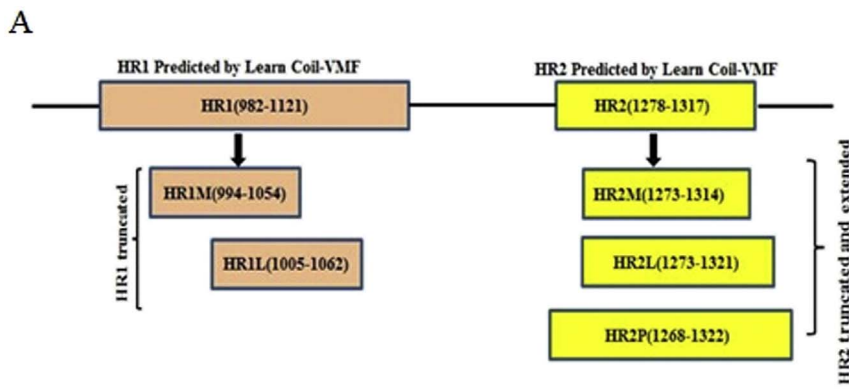
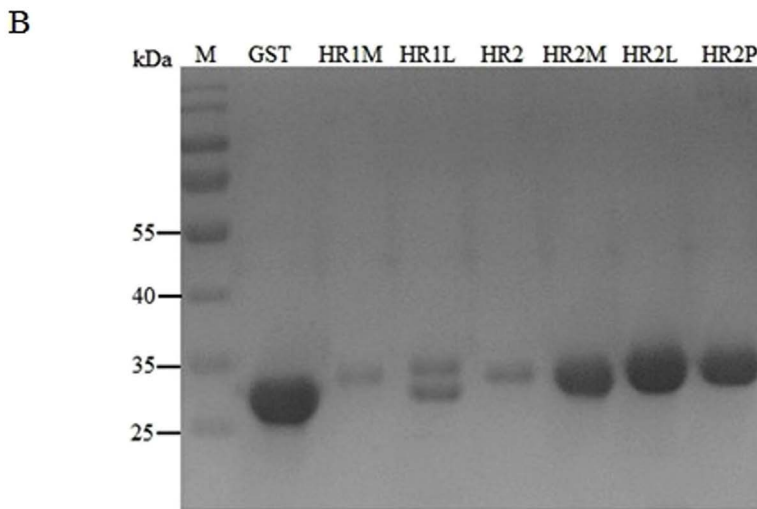


Fig. 2. Design and expression of peptides derived from PEDV S2 subunit HR1 and HR2 as GST-fusion proteins. (A) Design of six peptides based on the analysis of HR1 and HR2. (B) Expression and purification of GST-fusion proteins. M, protein molecular mass markers.



trypsin that might affect the integrity of the peptides, we use Huh7 cells as the *in vitro* model, instead of Vero cells, for the following virus inhibitory experiments. To confirm that the peptides did not have toxic effects on Huh7 cells, monolayers were exposed to a final concentration of 40  $\mu\text{M}$  of each peptide for 24, 48 or 72 h, and cell viability was analyzed using the MTT assay. There was no statistically significant difference between the viability of the control (untreated) cells and the cells exposed to the GST-fusion peptides or GST protein alone (Supplemental Fig. 1).

### 3.3. PEDV HR2-derived peptides exhibited antiviral activity *in vitro*

Based on the cell viability results, peptide concentrations of 5, 10, 20 and 40  $\mu\text{M}$  were chosen for testing of dose-dependence of PEDV infection inhibition in Huh7 cells, respectively. We first tested the effects of PEDV-peptide mixtures during the stage of PEDV-GFP inoculation (co-treatment). As shown in Fig. 3, HR1M and HR1L did not have inhibitory effects on PEDV-GFP infection in comparison with the GST control protein, whereas HR2 statistically suppressed PEDV-GFP infection at the concentrations of 20 and 40  $\mu\text{M}$ . In contrast, HR2M, HR2L and HR2P could significantly reduce the numbers of GFP positive cells at all tested doses (except 5  $\mu\text{M}$  for HR2M), with maximal reductions of 82%, 85% and 96% (40  $\mu\text{M}$ ), respectively. The deduced  $\text{IC}_{50}$  values of HR2M, HR2L and HR2P were 4.77, 2.96 and 1.11  $\mu\text{M}$ , respectively, and inhibition was concentration-dependent compared with untreated groups (Table 2). The HR2P peptide was the most potent inhibitor of PEDV-GFP virus replication among those tested. The results

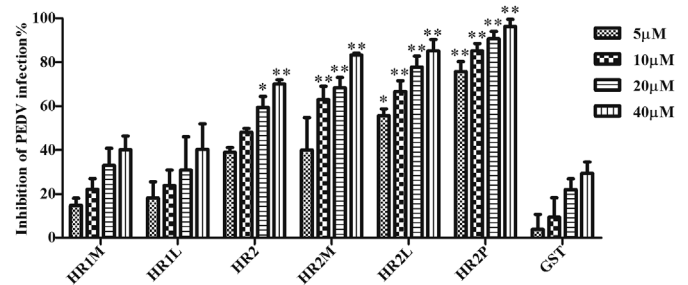


Fig. 3. Inhibition of PEDV-GFP virus by HR2-derived peptides. Huh7 cells were incubated with PEDV-GFP (MOI = 0.01) mixed with soluble HR peptides at various concentrations (5, 10, 20, and 40  $\mu\text{M}$ ). Viral infection was determined by counting GFP positive cell clusters. Figure represents mean  $\pm$  standard error of triplicate wells. The GST was used as the control for comparison. Significant (\*):  $p < 0.05$ ; Very significant (\*\*):  $p < 0.01$ .

indicated that the HR2-derived peptides target the HR2 domain in the PEDV S2 subunit to inhibit PEDV infection specifically.

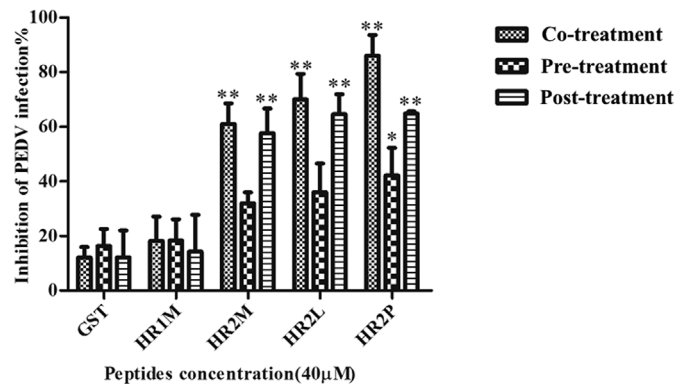
### 3.4. HR2L and HR2P have potent antiviral activities at different steps of viral infection and cross-reacted with G1 PEDV

An experiment was conducted to identify which steps in the entry process were inhibited by three HR2-derived peptides (HR2M, HR2L and HR2P) at a concentration of 40  $\mu\text{M}$  (the concentration which showed significant inhibition). The GST and HR1M peptide served as

**Table 2**  
Inhibitory effects of PEDV HR peptides.

Peptide	Highest concentration tested ( $\mu\text{M}$ )	IC <sub>50</sub> ( $\mu\text{M}$ )	Inhibition of viral titer (%)
GST	40	NA	29.4
HR1M	40	NA	40.20
HR1L	40	NA	40.30
HR2	40	12.98	70.04
HR2M	40	4.77	83.29
HR2L	40	2.96	85.15
HR2P	40	1.11	96.28

NA: Not analyzed.



**Fig. 4.** Inhibition of PEDV-GFP at different steps of viral infection. HR peptides (40  $\mu\text{M}$ ) were used to treat Huh7 cells before (pre-treatment), during (co-treatment), and after (post-treatment) infection with PEDV-GFP. The figure represents mean  $\pm$  standard error of triplicate wells. The GST was used as the control for comparison. Significant (\*):  $p < 0.05$ ; Very significant (\*\*):  $p < 0.01$ .

the control. Three different tests were conducted: cells exposed to peptides prior to infection (pre-treatment), during entry (co-treatment), or after viral entry (post-treatment). HR2M, HR2L, and HR2P showed potent antiviral activity in co-treatment, and to a lesser extent in other assays (Fig. 4). The HR2L and HR2P peptides suppressed the numbers of GFP-positive cells by about 60% at 40  $\mu\text{M}$  in post-treatment, whereas HR1M and the GST control inhibited by only 12–14% in all three methods (Fig. 4). These results indicate that treatment with HR2-derived peptides may serve as an antiviral intervention by suppressing PEDV entry.

The ability of the peptides to cross-react with a different PEDV strain representing G1 (strain ZJU/G1/2013) was also tested. Various peptides from the HR2 region of PEDV-GFP (HR2M, HR2L and HR2P) demonstrated antiviral activity against ZJU/G1/2013 in comparison with the GST control, reducing the numbers of PEDV-producing FFU by 20–62% when used in co-treatment, as determined by immunofluorescence assay (IFA) with an anti-PEDV-S1 antibody (Fig. 5A and B).

### 3.5. Neutralizing activity of the polyclonal antisera against HR2P

We further hypothesized that the HR2-derived antibody may also block virus entry as the HR2-derived peptides did. Therefore, we immunized mice with the soluble PEDV HR2P-His protein expressed in *E. coli*, and tested the reactivity of the resulting antisera. The serum antibody titers rose to 1:256,000, as determined by ELISA using HR2P-His as the antigen. Moreover, the antisera could recognize either HR2P-His or HR2P-GST protein at a 1:2000 dilution, whereas the pre-immunized sera could not react with them in western blot analysis (data not shown). The HR2P antisera could also cross-react with the soluble HR2, HR2M and HR2L proteins (data not shown). All of these results suggested that HR2P fragment was immunogenic and could elicit the

antibodies specific to the PEDV HR2 domain.

To determine whether immunization with HR2P-His could induce neutralizing antibodies against PEDV, we analyzed the neutralizing activity of the antisera with the VN assay using PEDV-GFP. As shown in Fig. 6, the pre-immunized serum could not inhibit PEDV-GFP infection. Previous work had shown that an anti-PEDV-S2 monoclonal antibody (mAb) recognizing the linear epitope GPRLQPY at the carboxy-terminal of the S2 subunit exhibited neutralizing activity against PEDV (Cruz et al., 2008). A similar anti-S2-mAb reacted with this epitope (a gift from Dr. Ying Fang, unpublished data) was used as the positive control, which suppressed about 50% of PEDV-GFP infection between 1:128 to 1:256 dilutions (Fig. 6). The antiserum against HR2P also showed neutralizing activity and could inhibit about 50% of PEDV-GFP infectivity at the 1:64 dilution (Fig. 6). The data demonstrated that the PEDV HR2P was highly immunogenic and may mimic a neutralizing epitope of PEDV, as its polyclonal antisera were able to efficiently block PEDV infection *in vitro*.

## 4. Discussion

As membrane fusion is of fundamental importance to the infection process of enveloped viruses, it is controlled by one or more viral surface proteins that must undergo conformational changes (Harrison, 2015). The use of HR peptides to interfere with fusion of the viral envelope to the host membrane is recognized to successfully inhibit the infection of viruses that have class I fusion proteins (Miller et al., 2011; Porotto et al., 2010). In the early 1990s, synthetic peptides derived from the HR2 domain of HIV-1 were discovered to be highly potent inhibitors of HIV fusion (Wild et al., 1994). This anti-HIV-1 peptide (T-20; brand name: Fuzeon; generic name: enfuvirtide) was licensed by the FDA in 2003, and has already been incorporated into clinical regimens with strong efficacy (Pang et al., 2009). In addition, the HR-derived peptides (HR1 and HR2) of MERS-CoV, SARS-CoV and FCoV can effectively inhibit viral membrane fusion, and though the crystal structure of the PEDV S protein has not been reported, similar regions were identified by amino acid sequence alignment. In this study, peptides corresponding to PEDV HR1 and HR2 were expressed in order to empirically confirm their function in PEDV.

It has been reported that peptides derived from HR2 domain in particular are able to inhibit infection of feline coronavirus (Liu et al., 2013), MERS-CoV (Lu et al., 2014), and SARS-CoV (Bosch et al., 2004). The inhibitory efficacy was not related to the length of the HR2 peptides, and the amino acid residues 1161–1175 were essential for inhibiting SARS-CoV infection. In the current work, the potential inhibitory activity of the HR1- and HR2-derived peptides was studied on PEDV replication *in vitro*. The antiviral and cross-reactivity of the soluble recombinant peptides were measured in three different ways. HR2M, HR2L, HR2P efficiently suppressed PEDV replication in both co- and post-treatments, with IC<sub>50</sub>'s less than 12  $\mu\text{M}$ . However, it was unknown why the HR2 peptides exerted impact in post-treatment. We hypothesized that binding of the peptides to the surface of infected and uninfected cells may restrict the re-infection and spread of the progeny viruses. Also, the PEDV inhibitory effect to a certain degree in HR2M, HR2L and HR2P pre-treatments might come from the presence of the peptides bound to the cellular surface non-specifically. Future studies on molecular dissecting of PEDV entry are warranted to answer this question. Analysis of antigenic characteristics of the peptides (Supplemental Fig. 2) indicated that HR2M, HR2L and HR2P exhibited strong hydrophilicity, surface accessibility, and flexibility, which are in line with the characteristics of immunogenic epitopes. While examining the inhibitory effect of these peptides, we found that HR2P, with extra C-terminal amino acids “NRVET” compared to HR2M and HR2L, showed significant inhibitory activity (Figs. 2 and 3). The difference in inhibition efficiency of HR2P, HR2M and HR2L indicates that the conserved C-terminus of PEDV HR2 may play an important role in viral invasion.



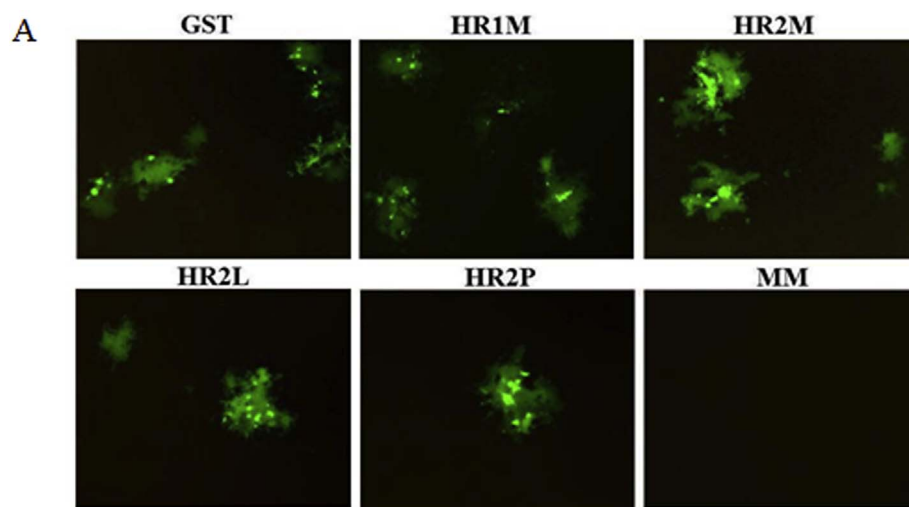


Fig. 5. Cross-reactivity of PEDV-GFP virus (G2 strain) HR2-derived peptides with PEDV G1 strain ZJU/G1/2013. (A) Representative IFA results. Cells were fixed at 48 hpi and incubated with a monoclonal antibody against the PEDV-S1 protein, followed by incubation with a FITC-conjugated goat anti-mouse secondary antibody. (B) Comparison of peptide inhibition of PEDV-G1 infection by quantification of PEDV-G1-producing FFU based on IFA results. The GST was used as the control for comparison. Significant (\*):  $p < 0.05$ ; Very significant (\*\*):  $p < 0.01$ .

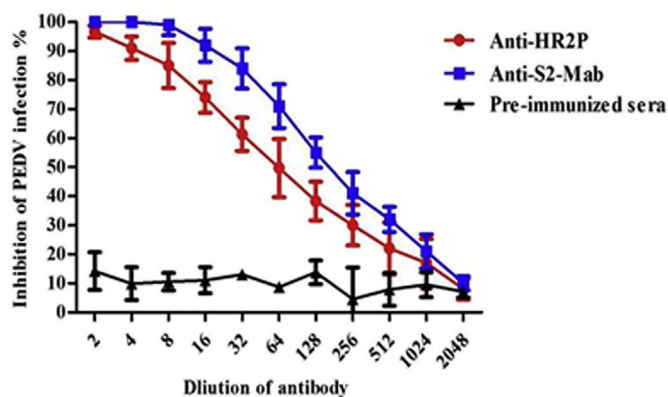
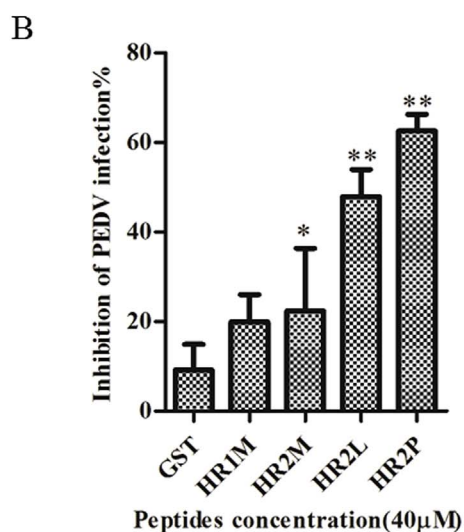


Fig. 6. The VN testing of mice anti-HR2P antisera. Huh7 cells were incubated with indicated mixture of PEDV (100 FFU/well) and dilutions of antisera, and reduction in FFU was measured. The figure represented mean values from three independent experiments, and error bars indicate standard deviation.

The spike glycoprotein is naturally found in trimeric structures on the surface of virus particles, and is responsible for adsorption and fusion while inducing host neutralizing antibodies and immune responses, highlighting its importance in vaccine development. The polyclonal antibodies induced by PEDV-HR2P linear antigens in this study demonstrated strong neutralization of PEDV *in vitro* (Fig. 6),

probably due to blockage of HR2-mediated virus membrane fusion. On the basis of this finding, better strategies of immunization may be devised for inducing neutralizing antibodies to PEDV with the HR2P sequence.

In summary, we designed, tested and characterized HR2-derived peptides that were able to inhibit PEDV entry and infection in cell culture. These peptides exhibit low cytotoxicity and biological function *in vitro*, while prepared anti-HR2P antibodies had neutralizing virus capacity. HR2-derived peptides could thus serve as a tool for dissecting the fusion mechanism of PEDV, as well as potential antiviral drugs, with broad application for the prevention and treatment of PEDV-associated diseases.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.antiviral.2017.11.021>.



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