# A Candidate Multi-epitope Vaccine Against Porcine Reproductive and Respiratory Syndrome Virus and *Mycoplasma Hyopneumoniae*

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**Research Article**

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# Abstract

**Background:** Porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*, Mhp) are two of the most common pathogens involved in the porcine respiratory disease complex (PRDC) which results in significant worldwide economic losses. Vaccination is reported to be the most effective approach to prevent the disease. Since PRRSV and Mhp co-infections are very common, an efficient dual vaccine against PRRSV and Mhp co-infections is required for the global swine industry. Compare with the traditional vaccine, the multi-epitope vaccines have several advantages, such as comparatively easy production and construction, chemical stability, and lack of infectious potential.

**Results:** In this study, to develop a safe and effective vaccine, B cell epitopes and T cell epitopes of PRRSV-GP5, PRRSV-M, Mhp-P46 and Mhp-P65 protein had been screened to construct a recombinant epitope protein rEP-PM which has good hydrophilicity, strong antigenicity and high surface accessibility, and each epitope is independent and complete. After immunization in mice, rEP-PM could induce the production of high levels of antibodies and effective cellular immune response.

**Conclusions:** Our results showed that the rEP-PM protein could be potential candidate to develop into a safe and effective multi-epitope peptide combined vaccine used in the control of PRRSV and Mhp infection.


# Background

Porcine reproductive and respiratory syndrome (PRRS), which is characterized by severe reproductive failure in sows, and respiratory distress in piglets and growing pigs, is one of the most economically significant swine diseases (1). The causative agent, PRRS virus (PRRSV), a positive-stranded enveloped RNA virus which belongs to the family *Arteriviridae* (2). *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*, Mhp) is the primary pathogen of enzootic pneumonia (EP), a chronic respiratory disease in pigs, can cause huge losses to the pig industry(3). Without co-infected with other pathogens, the main clinical outcome caused by Mhp is a cough with slight fever and anorexia(4). However, the immunosuppression caused by Mhp infection provides conditions for the secondary infections. Co-infection with other pathogens, such as PRRSV(5), porcine circovirus type 2 (PCV2)(6) and swine influenza virus (SwIV)(7) has been reported for decades.

PRRSV and Mhp are two of the most common pathogens involved in the porcine respiratory disease complex (PRDC), a most costly swine disease which is characterized by slow growth, fever, cough, and dyspnea in finishing pigs(8, 9). PRRSV plays a key role in PRDC modulating the host immune response and favouring secondary bacterial infections(10). Other studies have found that infection with Mhp potentiated and prolonged PRRSV-induced pneumonia clinically, macroscopically and microscopically(5). Since PRRSV and Mhp co-infections are very common in swine herds worldwide, an efficient dual vaccine against PRRSV and Mhp co-infections is required for the global swine industry.

Basing on the chemical approach to synthesize the identified B cell and T cell epitopes, epitope vaccines can induce specific immune responses(11). Compare with the traditional vaccine, the multi-epitope vaccines have several advantages, such as comparatively easy production and construction, chemical stability, and lack of infectious potential(11–13). Due to these advantages multi-epitope vaccines can be considered as a promising strategy to preventing virus spread. A multi-epitope vaccine containing 6 antigenic peptides of Hepatitis C Virus (HCV) stimulates neutralizing humoral and persistent cellular responses in mice(14). Multi-variant epitope ensemble vaccine comprising the four mutation variants of avian leukosis virus subgroup J (ALV-J) could induce high level of antibody titer in immunized SPF chicken for at least 126 days(15).

In this study, in order to develop an effective vaccine that can prevent both PRRSV and Mhp infection at the same time, we tried to prepare an epitope-based vaccine. Part of the B cell epitopes and T cell epitopes from PRRSV-GP5 protein, PRRSV-M protein, Mhp-p46 protein and Mhp-P65 protein were linked together by linkers to construct a recombinant epitope protein which named rEP-PM. After injected mice with purified rEP-PM, the mice produced ideal humoral and cellular immune response. Hence, the epitope-based vaccine rEP-PM is a promising candidate vaccine against PRRSV and Mhp.

# Results

### Construction and characterization of recombinant epitope protein

We screened some B cell epitopes and T cell epitopes of the GP5 and M protein of PRRSV identified in other studies(16–18), according to the location and size of the epitopes, some of the epitopes have been selected as candidate epitopes and all selected epitopes were listed in Table 1. B cell epitopes and T cell epitopes of P46 and P65 protein of Mhp were predicted with Bepipred Linear Epitope Prediction and ProPred-I. According to the position and length of the epitopes, several candidate epitopes were screened out for the construction of recombinant protein (Table 2).

Table 1
Candidate epitopes of GP5 and M protein of PRRSV.

| Type of epitope | Protein | Sequence of amino acid |
|---|---|---|
| B cell | GP5 | SSHIQLIYNLTLCELNG |
| B cell | GP5 | VEKGGKVEVEGHLIDLKRV |
| T cell | GP5 | LAALICFVIRLAKNC |
| T cell | GP5 | RLYRWRSPV |
| B cell | M | FVVRRPGSTTVNGTLVPGLKSLVLGGR |
| B cell | M | AVKQGVVNLVKYAK |
| T cell | M | CNDSTAPQKVLLAFS |
| T cell | M | FGYMTFVHFESTNRV |

Table 2
Candidate epitopes of P46 and P65 protein of Mhp.

| Type of epitope | Protein | Sequence of amino acid |
|---|---|---|
| B cell | P46 | ANLSPAPKGFIIAPENGSGVGTA |
| B cell | P46 | KYDNQTYKVQGK |
| T cell | P46 | KKFLYSSAIYA |
| T cell | P46 | DLSPEGENAVYV |
| B cell | P65 | DQEEKDDSNAEELKNTTNFDDFDENKPTYS |
| B cell | P65 | STFDTDQEAAIKDDKRT |
| B cell | P65 | NKVKDYAR |
| T cell | P65 | GYKKIAHQLLLKLTLDQEEK |
| T cell | P65 | VREIVSLPIFDNFDFRELIPVKNPFV |

Candidate epitopes were concatenated together to construct the recombinant epitope protein rEP-PM, the linker KK had been used between T-T and T-B epitopes, GGGGS had been used to connect B-B epitopes (Fig. 1A). The hydrophilicity plot, antigenic index, surface probability plot and flexible regions were analyzed by using the Kyte-Doolittle, Jameson-Wolf, and Emini algorithms, respectively, with the aid of DNASTAR package (Fig. 1B). The results indicate that rEP-PM has good hydrophilicity, strong antigenicity and high surface accessibility, and each epitope is independent and complete.

The secondary structure of rEP-PM was predicted by SOPMA server, the result showed that rEP-PM consists of 21.51% alpha helix (h), 27.91% extended strand (e), 12.5% beta turn (t) and 38.08% random coil (c) secondary structural elements (Fig. 1C).

## Expression and purification of the rEP-PM

DNA sequence encoding rEP-PM was synthesized and cloned into the plasmid pET-28a, the supernatant and cell lysate were derived from *E.coli* BL21 transformed with pET-28a-rEP-PM and analyzed by western blot. A single protein band with a molecular mass of approximately 41kDa could be observed by anti-His monoclonal antibodies in the supernatant and cell lysate, no specific protein band has been detected in control group (Fig. 2A). This result showed that the rEP-PM was partially expressed in soluble form. Then the soluble rEP-PM in supernatant of *E.coli* BL21 has been purified with nickel affinity chromatography column (Fig. 2B). All these results demonstrated that the rEP-PM was successfully expressed and purified.

## rEP-PM could induce the production of high levels of antibodies in mice

To investigate whether rEP-PM could induce specific antibodies, four-weeks-old female BALB/c mice were divided into four groups of twelve mice in each group, mice were immunized as described in *Materials and methods.* Serum samples were taken from the twelve mice in each group at day 7, 14, 28 and 56 after the third immunization, the levels of antibodies in the serum of mice were assessed with indirect ELISA, the antibody titers of mice immunized with rEP-PM were higher than 12800 at day 7 (Fig. 3A), 14 (Fig. 3B), 28 (Fig. 3C) and 56 (Fig. 3D) after the third immunization. These results indicate that mice immunized with rEP-PM could induce high levels of antibodies, which maintained at high levels for a long time (8 weeks) after the last immunization.

## rEP-PM has good immunoreactivity

The specific immunoreactivity of rEP-PM in mice was further confirmed by western blot. 10 μg of purified rEP-PM was subjected to polyacrylamide gel electrophoresis, the serum of mice immunized with rEP-PM protein was used as the primary antibody and the goat anti-mouse IgG as the secondary antibody. The result indicate that rEP-PM could react specifically with antibodies induced by rEP-PM (Fig. 4A).

To further explore whether rEP-PM could react with anti-PRRSV antibodies and anti-Mhp antibodies, 10 μg of purified rEP-PM was subjected to polyacrylamide gel electrophoresis, the serum of mice immunized with PRRSV attenuated vaccine (containing anti-PRRSV antibodies) and Mhp-168 attenuated vaccine (containing anti-Mhp antibodies) were used as primary antibodies, respectively, and the goat anti-mouse IgG was used as the secondary antibody. As shown in Fig. 4B and 4C, the recombinant protein rEP-PM could react specifically both with anti-PRRSV (Fig. 4B) and anti-Mhp (Fig. 4C) antibodies. These data suggest that rEP-PM has good immunoreactivity with anti- rEP-PM, anti-PRRSV and anti-Mhp antibodies.

## rEP-PM could induce Th1 and Th2 cellar immune response in mice

To investigate whether rEP-PM could induce Th1 and Th2 cellular response, the concentration of IL-2 (which induced by Th1 cellular response) and IL-10 (which induced by Th2 cellular response) in the medium supernatant of splenic lymphocytes were detected by ELISA. As shown in Fig. 5, compared with the negative control group, both the level of IL-2 and IL-10 in the medium supernatant of splenic lymphocyte of the immunized groups was significantly higher than the negative control group. These results suggest that immunized with rEP-PM could induce both Th1 and Th2 cellular immune response in mice.

# Discussion

PRDC is one of the main causes of economic losses for swine producers worldwide. This complex is due to a combination of different pathogens and their interactions, Mhp and PRRSV are the two main pathogens involved in PRDC(19). Mhp and PRRSV are often infected simultaneously, infection with Mhp could potentiate and prolong PRRSV-induced pneumonia clinically(5). Vaccination is generally considered to be the most effective method of preventing infectious diseases. As a new and safe vaccine, epitope-based vaccine can induce a specific immune response to protect the host from pathogens. Compared with traditional vaccines, epitope-based vaccines have the advantages of low cost, multivalence, no genetic components, high antigen presentation efficiency, and convenient application(20, 21). Therefore, we explored the use of genetic engineering technology to efficiently express a recombinant protein, which named rEP-PM, containing multiple B cell epitopes and T cell epitopes of PRRSV and Mhp, and then evaluate the immunogenicity of rEP-PM.

In the design of epitope-based vaccines, both humoral immunity and cellular immunity need to be considered(22). B cell epitopes are necessary to stimulate the body to produce humoral immunity, in order to enhance the immune effect, the assistance of T cell epitopes which recognized by T cells is needed. The selection of a single epitope may cause immune failure due to mutations. Therefore, we choose two proteins for each pathogen, and at least two B cell epitopes and two T cell epitopes for each protein. The envelope glycoprotein GP5 and the non-glycosylated membrane protein M are major structural proteins of PRRSV virion, the epitopes of GP5 and M proteins we selected have been verified by previous studies(16, 17, 23, 24). The P46 and P65 proteins of Mhp are two membranous proteins carrying species-specific antigenic determinants, since fewer epitopes of Mhp-P46 and Mhp-P65 have been identified, we used Bepipred Linear Epitope Prediction to predict the B cell epitopes and ProPred-I to predict the T cell epitopes of Mhp-P46 and Mhp-P65. Finally, the rEP-PM we constructed contains two B epitopes and two T epitopes of PRRSV-GP5, two B epitopes and two T epitopes of PRRSV-M, two B epitopes and two T epitopes of Mhp-P46, three B epitopes and two T epitopes of Mhp-P65.

In order to allow each epitope to maintain independent structure and function, it is necessary to add linker or spacer between different candidate epitopes. The KK sequence is the target sequence for lysosomal proteolysis, and the lysosomal protein is an important protease in the MHC II antigen presentation pathway. Studies have shown that the T epitopes and the B epitopes are connected by KK, which is conducive to protease cutting between epitopes to make epitopes easier to be identified and

presented(25). GGGGS is a flexible peptide sequence, which is currently the most widely used linker sequence and is suitable for most epitope linkages(26). Therefore, we use KK between T-T and T-B epitopes, and GGGGS between B-B epitopes in the construction of rEP-PM. After analysis, the recombinant epitope protein we constructed is not easy to form a secondary structure, and it is difficult to form a new epitope.

After immunization with rEP-PM, the antibody level was high and lasted for a long time. After 8 weeks of immunization, there were still high levels of antibodies in the mice. Both the serum of mice immunized with the commercial PRRSV vaccine and the serum of mice immunized with the commercial Mhp vaccine can react with the rEP-PM, which shows that the B cell epitopes of rEP-PM are partly or fully included in the B cell epitopes which could stimulate the body to produce humoral immune response in the commercial PRRSV vaccine and Mhp vaccine. T lymphocyte cells play an important role in the generation of the pathogen-specific adaptive immune responses, due to the epitopes presented by antigen presenting cells could be recognized by T lymphocyte cells, which provided help for B lymphocyte cells to produce large quantities of antibodies. The results of rEP-PM-specific T lymphocyte proliferative response showed that rEP-PM vaccines could induce high levels of production of IL-2 and IL-10, suggesting that immunized with rEP-PM vaccine could induce both Th1 and Th2 cellular immune response in mice.

Overall, we have developed a recombinant epitopes vaccine, rEP-PM, which contained multiple B cell epitopes and T cell epitopes of PRRSV-GP5, PRRSV-M, Mhp-P46 and Mhp-P65 proteins. The rEP-PM vaccine could induce robust humoral immune response and cellular immune response in mice. Therefore, the rEP-PM vaccine may be potential candidate used in the control of PRRSV and Mhp infection.

## Conclusion

This study showed that recombinant epitopes vaccine rEP-PM could induce the production of high levels of antibodies and effective cellular immune response. rEP-PM protein could be potential candidate to develop into a safe and effective multi-epitope peptide combined vaccine used in the control of PRRSV and Mhp infection.

## Methods

# Animals and antibodies

- Female, 4-week-old, BALB/c mice were purchased from the Hebei Medical University and housed in the animal facility of the College of Life Science, Hebei Normal University. All of the animal experiments were approved by the Research Ethics Committee, Hebei Normal University, Hebei, China and in accordance with the Guide for the Care and Use of Laboratory Animals from Research Ethics Committee, Hebei Normal University.
- Additionally, anti-His tag, HRP-conjugated goat anti-mouse IgG used in the detection of purified rEP-PM and immunoreactivity of rEP-PM were purchased from Abcam (UK).

# PRRSV and Mhp

- PRRSV attenuated vaccine (Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Vaccine, Live, JXA1-R Strain) purchased from Heyuan Bioengineering Co., Ltd, Jilin, China. Mhp-168 attenuated vaccine purchased from Nanjing Tech-Bank Bio-industry Co., Ltd., Jiangsu, China.

# Detection of purified rEP-PM by western blot

- The DNA sequence encoding the rEP-PM was synthesized and cloned into the plasmid pET-28a. After DNA sequencing, the recombinant plasmid pET-28a- rEP-PM was transformed into *E.coli* BL21, cultured for 20h at 20°Cand induced with 0.5 mM IPTG. After disruption, supernatant was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 5% skim milk in Tris Buffered saline with Tween (TBST) (Solarbio, China) and incubated with anti-His monoclonal antibodies (1:5000) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Abcam, UK) (1:5000). After washing with TBST for three times, protein bands were detected using the enhanced chemiluminescence system and analyzed using the Image Lab software 4.0.1.

# Detection of immunoreactivity of rEP-PM by western blot

- 10μg of purified rEP-PM was subjected to SDS-PAGE, and then transferred onto PVDF membranes. After blocking with TBST containing 5% BSA Blocking Buffer (Solarbio, China), the membranes were washed with TBST thrice and incubated with serum of mice immunized with rEP-PM, PRRSV attenuated vaccine and Mhp attenuated vaccine (1:5000), respectively, then incubated with HRP-conjugated goat anti-mouse IgG (1:5000). After wash with TBST for three times, the protein bands were detected using the enhanced chemiluminescence system and analyzed using the Image Lab software 4.0.1.

# Immunization of mice

- Forty-eight female BALB/c mice (4-weeks old each) were randomly divided into four groups (1–4) with twelve mice per group. Mice in group 1 (negative control group) were injected intraperitoneally with 0.1mL of normal saline, mice in group 2 were injected with 0.1mL PRRSV attenuated vaccine intramuscularly, mice in group 3 were injected with 0.1mL Mhp-168 attenuated vaccine intramuscularly, mice in group 4 were intramuscularly vaccinated with 0.1mL of the solution containing 50 μg of rEP-PM protein emulsified with Freund's adjuvant (Solarbio, China). All the mice were injected with the same dose of vaccine as booster immunization at 7 days post-primary immunization, only the mice in group 3 immunized subcutaneously, the injection methods of the other groups were the same as the first immunization. All the mice were injected with the same dose of vaccine in the same methods with the primary immunization as booster immunization at 14 days post-primary immunization. Serum samples were collected at 7, 14, 28 and 56 after the third immunization and stored at -20°C until use.

# Antibodies detection using indirect ELISA

- Serum was collected at day 7, 14, 28 and 56 after the third immunization to detect the level of antibodies in mice. Antibodies titers against rEP-PM, PRRSV and Mhp were measured respectively by indirect ELISA. Serum of normal saline immunized mice was used as negative control. The 96-wells ELISA plate was coated with rEP-PM, Mhp culture lysate or PRRSV culture lysate (2 μg/mL, 100 μL/well) at 4°C overnight. After blocking with 2% BSA (300 μL/well) at 37°C for 2h and washing by PBST, serum dilution (1:100; 1:200; 1:400; 1:800; 1:1600; 1:3200; 1:6400; 1:12800) of different groups were added respectively, then incubated at 37°C for 1h, as well as the negative control. After washing for three times, HRP-conjugated goat anti-mouse IgG (1:5000, 100μl/well) was added and incubated for 1 h at 37°C. Plates were then incubated with the 3,3',5,5'-tetramethylbenzidine (TMB) substrate and the optical density (OD) was measured at 450 nm (O.D.450 nm).

# Analysis of IL-2 and IL-10 expression though ELISA assay

- Splenic lymphocytes were isolated and resuspended in RPMI 1640 medium supplemented with 10% FBS. Cell densities were adjusted to 4×106 cells/mL and plated in a 12-well flat-bottom tissue culture plate with 10 μg/mL purified rEP-PM. After 72 h of incubation at 37 ℃, the cell-free supernatant was removed and detected using commercially available mouse interleukin (IL)-2, and interleukin (IL)-10 ELISA kit (Dakewei, China) in accordance with the manufacturer's protocol. The concentrations of different cytokines were determined by the standard curve.

# Statistical analysis

- Statistical analysis was performed using Prism GraphPad 5.0 software (GraphPad Software, USA). Data are presented by mean ± SEM. P values were determined by Student's t-test. Differences were considered statistically significant at $p < 0.05$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

## Abbreviations

ELISA: Enzyme linked immunosorbent assay; HRP: Horseradish peroxidase; Mhp: *Mycoplasma hyopneumoniae*; OD: Optical density; PRDC: porcine respiratory disease complex; PRRSV: Porcine reproductive and respiratory syndrome virus; SD: Standard deviations; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST: Tris Buffered saline with Tween;

## Declarations

- **Ethics approval and consent to participate**

This study was conducted according to the guidelines of the Care and Use of Laboratory Animals from Research Ethics Committee, and approved by the Research Ethics Committee of Hebei Normal University (Approval No.: 2019-077)

- **Statement**

The study was carried out in compliance with the ARRIVE guidelines.

- **Consent for publication**

Not applicable.

- **Availability of data and materials**

All data generated or analysed during this study are included in this published article.

- **Competing interests**

The Authors do not have any conflicts of interest.

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- **Authors' contributions**

Designed the experiments: ZG, BZ and RZ; Performed the experiments: ZG, LC, XP, and XL; Analyzed the data: ZG, GL, YT, XW and RZ; Wrote the paper: ZG and RZ; Proofed the manuscript: ZG, LC, XP, XL, GL, YT, XW, BZ and RZ. All authors read and approved the final manuscript.

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Not applicable

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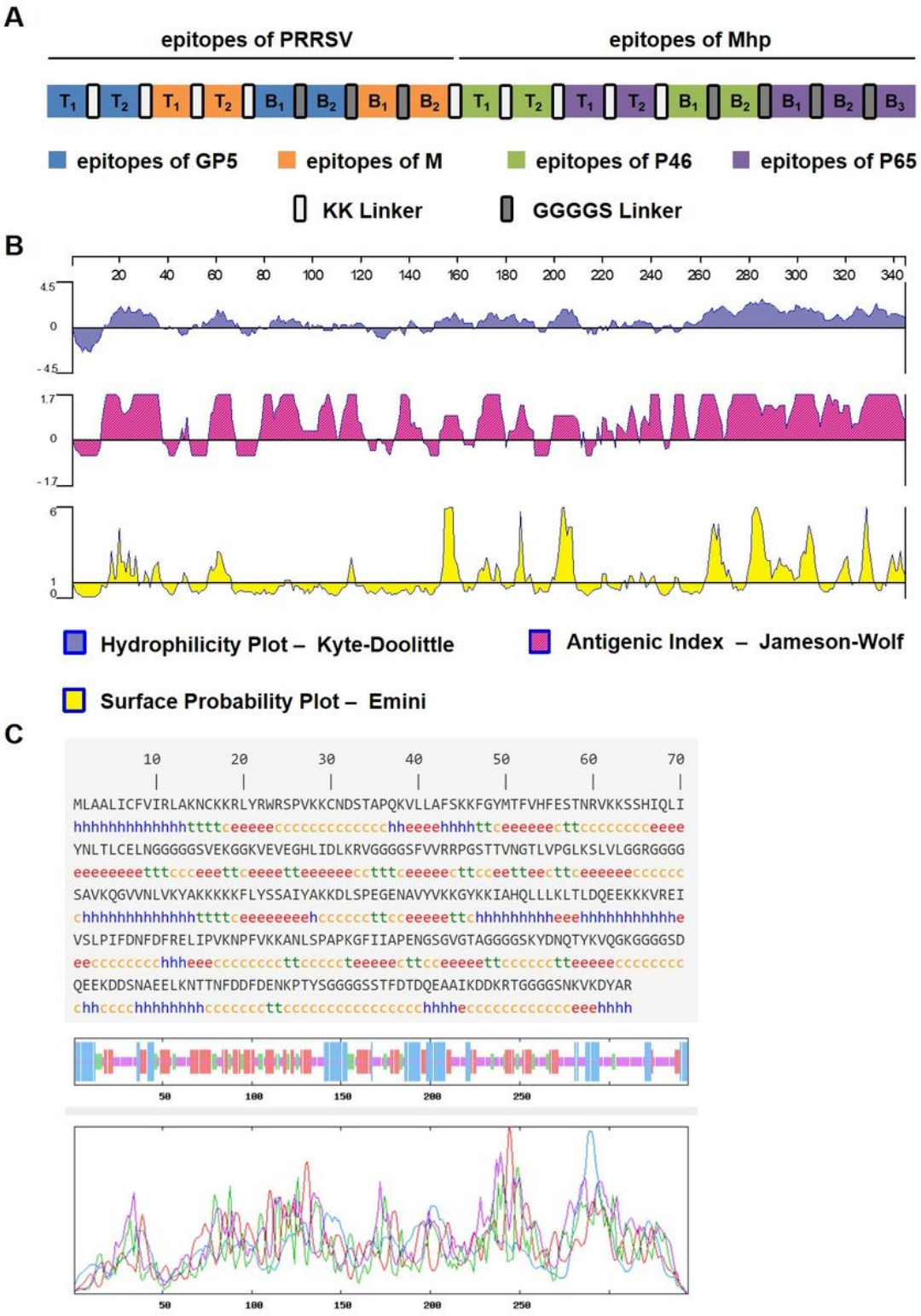
# Figures




## Figure 1

Construction and characterization of recombinant epitope protein. (A) Schematic diagram describing the construction of candidate epitopes. (B) The hydrophilicity plot, antigenic index and surface probability plot of rEP-PM. The region from 0 to 4.5 in the hydrophilicity plot indicates that the protein is hydrophilic, and the region from -4.5 to 0 indicates that the protein is hydrophobic. The region 0 to 1.7 in the antigenic index indicates that the antigenicity is good, and the region -1.7 to 0 indicates that the antigenicity is poor.

The area ≥1 in the surface probability plot represents the surface accessibility area of the protein, and the area ≤1 represents the surface inaccessibility area of the protein. (C) The predicted secondary structure of rEP-PM. The blue h represents alpha helix, the red e represents extant strand, the green t represents beta-turn and the yellow c represents random coil.

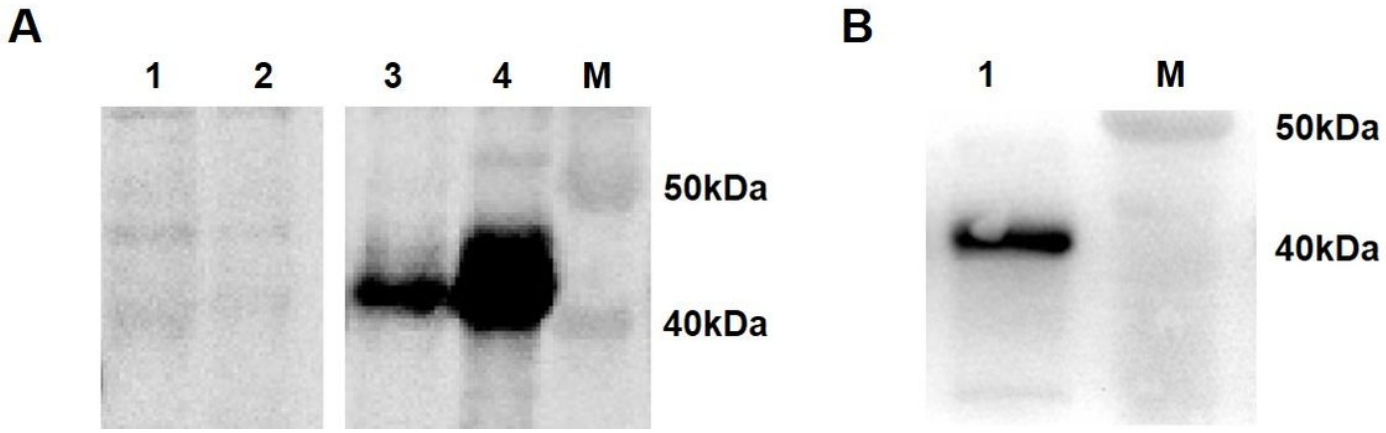
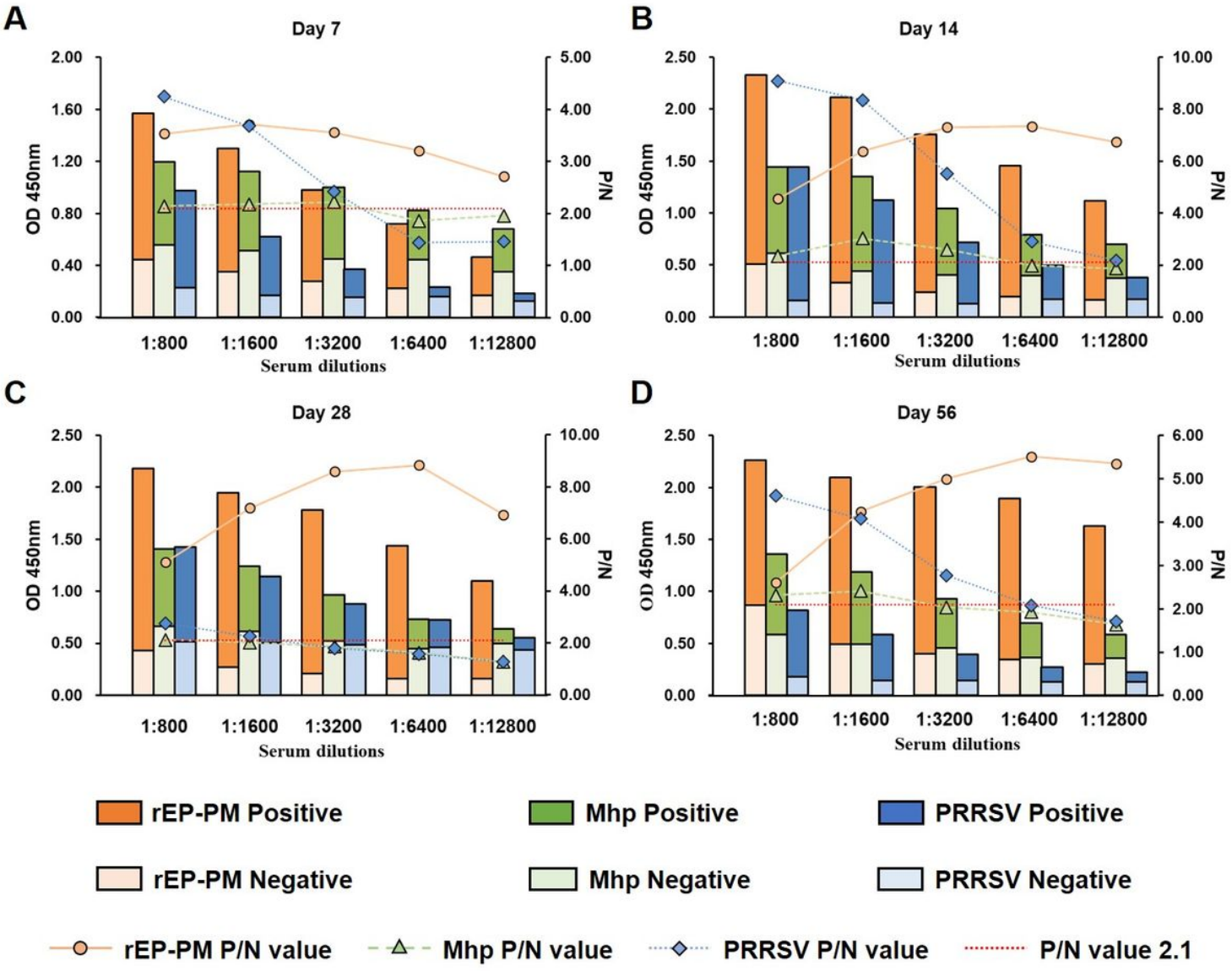



**Figure 2**

Expression and purification of the rEP-PM. (A) Western blot analysis of rEP-PM expressed in E.coli BL21 transformed with pET-28a-rEP-PM. Lane 1: Culture supernatant of E.coli BL21 transformed with pET-28a; Lane 2: Lysates of E.coli BL21 transformed with pET-28; Lane 3: Culture supernatant of E.coli BL21 transformed with pET-28a-rEP-PM; Lane 4: Lysates of E.coli BL21 transformed with pET-28a-rEP-PM; M: Protein marker. (B) Western blot analysis of purified rEP-PM. Lane 1: The rEP-PM purified from culture supernatant of E.coli BL21 transformed with pET-28a-rEP-PM; M: Protein marker. Uncropped blots are presented in Supplementary Figure 1.




## Figure 3

rEP-PM could induce the production of high levels of antibodies in mice. The level of anti-rEP-PM, anti-PRRSV and anti-Mhp of immunized mice(n=12) in different groups was detected by indirect ELISA at day 7 (A), 14 (B), 28 (C) and 56 (D) after the third immunization. The P/N (OD 450nm value of positive serum/OD 450nm value of negative serum) values were shown on the right Y axis, P/N value (>2.1) was determined to be positive results.

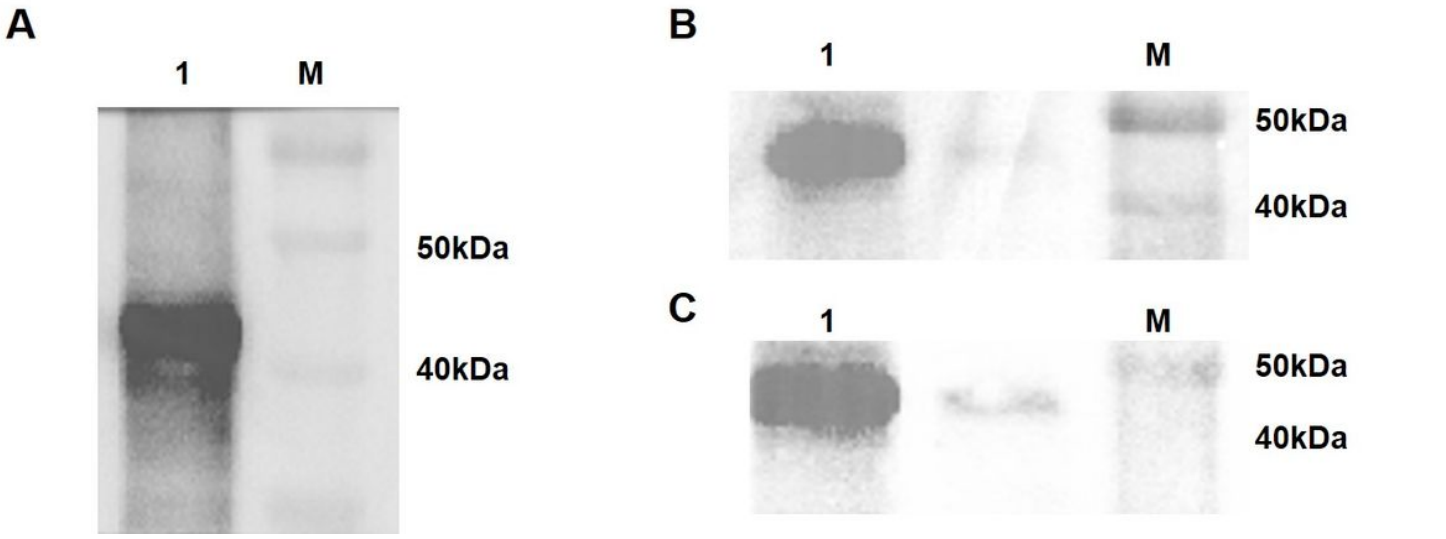



Figure 4

rEP-PM has good immunoreactivity. 10 μg of rEP-PM was subjected to SDS-PAGE and then incubated with serum of mice immunized with rEP-PM (A), PRRSV attenuated vaccine (B) and Mhp attenuated vaccine (C), respectively. HRP-conjugated goat anti-mouse IgG (1:5000) was used as secondary antibody. M: Protein marker. Uncropped blots are presented in Supplementary Figure 2.

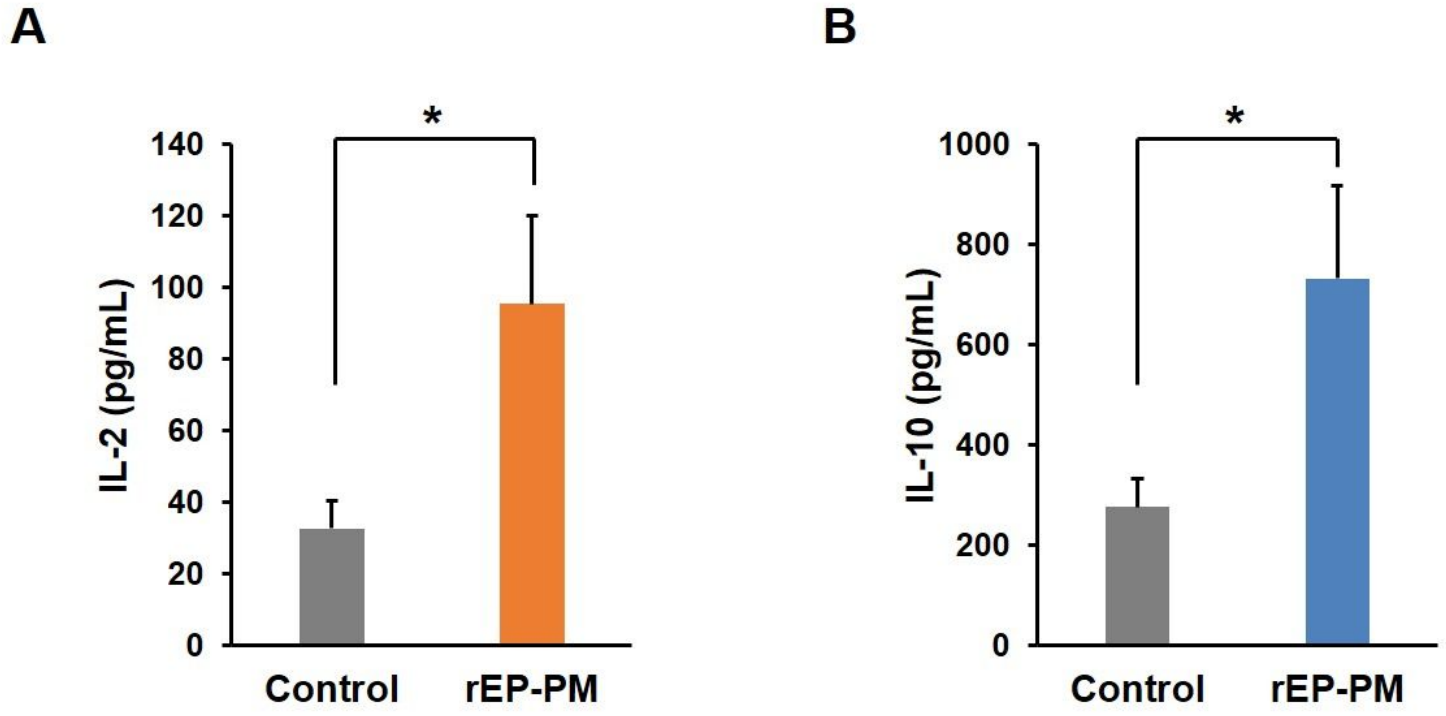



Figure 5

rEP-PM could induce Th1 and Th2 cellar immune response in mice. IL-2 (A) and IL-10 (B) cytokine production in the supernatants of splenic lymphocytes stimulated with rEP-PM from mice immunized with rEP-PM or normal saline (negative control) were detected with commercial ELISA kits according to

the manufacturer’s instruction (n=5). Data are presented by mean ± SEM. P values were determined by Student's t-test. * P< 0.05; ** P< 0.01; *** P< 0.001.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.jpg
- SupplementaryFigure2.jpg
- Figurelegendsforthesupplementaryfigures.docx