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STUDY ON THE PRODUCTION OF IMMUNOGENIC S ANTIGEN FROM PORCINE EPIDEMIC DIARRHEA VIRUS (PEDV) IN *NICOTIANA BENTHAMIANA* FOR THE DEVELOPMENT OF A NEW GENERATION VACCINE

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INTRODUCTION

The importance of the thesis

Porcine Epidemic Diarrhea (PED) is a dangerous disease with the potential to spread among pigs of all ages. The Porcine Epidemic Diarrhea Virus (PEDV), a virus belonging to the Coronaviridae family, is the causative agent of PED [1]. PEDV strains belonging to group G2 have had a significant impact on the economy, reducing the productivity of sows and piglets in many provinces and cities

The differences in the gene sequences of new outbreaks of PEDV strains and the PEDV strains in vaccines, especially in neutralizing epitopes, lead to the current vaccines having low protective efficacy against field PEDV strains [3]. Subunit vaccines are produced using a plant-based system, which offers several advantages including low production costs, easy scalability, high stability, and long shelf life [6]. The transient expression method based on agro-infiltration has the advantage of quickly producing large quantities of recombinant proteins, enabling timely responses to disease outbreaks [7].

The PEDV S protein is an essential factor in the virus's interaction with cellular receptors [8] and is considered the primary target for vaccine development [9]. The CO-26K equivalent region (COE) (aa 499-638), the S1D region (aa 636-789), and the S2 subunit are recognized as antigenic regions on the S protein [14].

Till now, the expression of the COE region (aa 499-638), COE-S1D region (aa 499-789), or S2 region (aa 730-1324) within the S protein fused with the GCN4pII motif (abbreviated as pII) or the GCN4pII-tp motif (abbreviated as pII-tp) on *N. benthamiana* plants and the immunogenicity of these proteins in experimental animals has not been demonstrated.

Based on scientific principles and practical considerations, we have undertaken the research project: '**Study on the production of immunogenic S antigen from Porcine epidemic diarrhea virus (PEDV) in *Nicotiana benthamiana* for the development of a new generation vaccine**'.

The research objectives of the thesis

Produce a recombinant S antigen of PEDV from *Nicotiana benthamiana* plants and evaluate its immunogenicity in experimental animals to support vaccine development.

The main research contents of the thesis

Content 1: Production and immunogenicity evaluation of the COE/G1a-pII recombinant antigen from a PEDV strain of group G1a.

Content 2: Production and immunogenicity evaluation of the COE/G2a-pII recombinant antigen from a PEDV strain of group G2a causing disease in Vietnam.

Content 3: Production and immunogenicity evaluation of the recombinant antigens COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII from a PEDV strain of group G2a causing disease in Vietnam.

The scientific and practical significance of the thesis

In Vietnam, PEDV strains belonging to group G2 are currently the primary causative agents of PED outbreaks. Developing an effective vaccine to prevent the PEDV strains of group G2 in Vietnam is of very high importance. The research results of this thesis demonstrate that recombinant proteins produced in *Nicotiana benthamiana* plants using transient expression technology such as COE/G2a-pII, COE-S1D/G2a-pII, and COE/G2a-pII-tp exhibit high antigenicity, strong immunogenicity, and hold great promise as vaccine candidates for PED prevention in Vietnam.

Significant contributions of the thesis

The thesis is systematic research on the production of COE antigens from attenuated PEDV strain DR13 belonging to group G1a, and COE, COE-S1D, and S2 antigens from the highly virulent NAVET/PEDV/PS6/2010 strain belonging to group G2a, which causes endemic diseases in Vietnam.

These antigens incorporating the pII motif or pII-tp motif were produced in *Nicotiana benthamiana* plants using transient expression. Furthermore, the immunogenicity of the recombinant antigens was assessed in experimental animals.

The COE/G2a-pII antigen induced immune response to protect piglets born to the COE/G2a-pII-vaccinated sow (100µg/dose) against PEDV after the virulent challenge. The COE-S1D/G2a-pII antigen (50µg/dose) combined with Emulsigen®-D (MVP) adjuvant stimulated stronger PEDV-specific IgG and PEDV-specific IgA antibodies that were of capable neutralizing PEDV, equivalent to the commercial Corning vaccine (Wuhan Keqian Biology). These findings suggest that these antigens are potential candidates for the production of subunit vaccines against PEDV in Vietnam.

Chapter 1. LITERATURE REVIEW

1.1. Porcine epidemic diarrhea

1.1.1. The global situation of PED outbreaks

Since PED first reported occurrence in the United Kingdom and Belgium in 1970, its outbreaks have continued to be detected in many other countries across Europe, Asia, and the Americas.

1.1.2. The situation of PED outbreaks in Vietnam

Until now, PED has appeared in all major pig farming regions in Vietnam.

1.2. Causative agent of PED

1.2.1. Classification of PEDV

PEDV is classified in the Coronaviridae family (subfamily Coronavirinae, family Coronaviridae, order Nidovirales) [37].

1.2.2. Morphological Characteristics of PEDV

PEDV particles exist in a spherical form with a diameter ranging from 90 to 160 nm under electron microscopy.

1.2.3. Structural characteristics of the genome and functions of PEDV proteins

PEDV has a single-stranded RNA genome with a size of approximately 28 kb [37], which contains 7 open reading frames (ORFs) encoding 4 structural proteins of PEDV, namely N (Nucleocapsid), S (Spike), E (Envelope), M (Membrane), and three non-structural proteins, including ORF 1a, 1b, and ORF3.

1.2.4. The S protein of PEDV

The regions containing antigenic determinants recognized by B-cell receptors and reacting with neutralizing antibodies on the S protein include the N-terminal domain [NTD]/S0 (aa 19-202), the CO-26K equivalent (COE) (from aa 499 to 638), S1D (from aa 636-789, recognized by two B-cell receptors, SS2 (aa 748-755) and SS6 (aa 764-771)), and 2C10 (aa 1368-1374, aa 1371-1377) [8, 14, 15, 22]. These regions containing antigenic determinants on the S protein are considered primary candidates for subunit vaccine development against PEDV [47].

1.2.5. Molecular epidemiology of PEDV strains worldwide

Studies on the genetic characteristics based on the S gene have shown that PEDV can be classified into 2 groups: the classical G1 group and the epidemic G2 group. The G1 and G2 groups can further be divided into subgroups, namely G1a, G1b, G2a, and G2b. PEDV can also be categorized into 2 types: non-S INDEL and S INDEL, based on insertions or deletions in the S gene [52].

1.2.6. Molecular epidemiology of PEDV strains in Vietnam

Twenty-eight PEDV strains were collected in Northern and Central Vietnam, belonging to group G2 (both subgroups G2a and G2b), while two other PEDV strains (HUAPED176 and HUA-PED254) were collected in the Southern region, belonging to group G1 (subgroup G1b) [4].

1.3. Vaccines for preventing PEDV

1.3.1. Inactivated vaccine and live attenuated vaccine

Some inactivated and live attenuated vaccines have been licensed and commercialized in China, Japan, and South Korea. These types of vaccines have been demonstrated to provide protection in laboratory animal conditions, but their effectiveness in practical applications is still a subject of debate.

1.3.2. Subunit vaccine

In recent years, a portion of the PEDV S protein, including COE, S1 or the entire S protein, has been expressed in systems such as *E. coli*, *Bacillus subtilis*, Baculovirus, Adenovirus, *Lactobacillus*, and plants. Mice or pigs immunized with these vaccines via oral, intramuscular, subcutaneous, or intraperitoneal routes have stimulated the production of high levels of IgG and IgA antibodies.

1.3.3. Nucleic acid vaccine

The mRNA-based PEDV vaccine using an alphavirus vector carrying the PEDV S gene from the company Harrisvaccines in the United States was conditionally licensed by the United States Department of Agriculture in 2014.

1.3.4. Challenges in the development of PEDV vaccines

At present, PEDV vaccines may not effectively prevent PEDV outbreaks due to the frequent mutations of the virus, and these vaccines may not generate sufficient mucosal immunity.

1.4. Plant-derived subunit vaccines

1.4.1. Transient expression system in plants

Compared to stable expression, transient expression in plants is receiving more attention due to its flexibility and rapid production of large quantities of recombinant bio-pharmaceutical proteins [103]. *N. benthamiana* is the most commonly used plant species for transient expression.

1.4.2. Plant-based vaccines for PEDV prevention worldwide

Most studies focus on the expression of the COE region of the S protein. The entire S protein has been reported to have low expression levels [18] or not expressed at all [19]. Tobacco plants are the subject of choice in the majority of studies for producing COE protein with high expression levels. COE protein accumulates to high levels in plants when transiently expressed.

1.4.3. GCN4pII motif and the tailpiece motif of IgM

1.4.3.1. GCN4pII motif

The GCN4-pII is an artificial motif designed and created by a core composed entirely of beta-branched residues. GCN4-pII is used to induce trimerization of the target proteins, simultaneously increasing their stability, solubility, and potentially tripling their size.

1.4.3.2. Tailpiece motif of IgM

The tailpiece (tp) is a short peptide sequence consisting of 18 amino acids (PTLYNVSLVMSDTAGTCY) at the C-terminus of IgM [139]. The peptides of tp play a crucial role in the polymerization process of IgM[140], including the formation of intramolecular disulfide bonds involving Cys575, the amino acid near the end of the tp [141]. The formation of pentameric/hexameric forms is controlled by mechanisms involving Cys575. Tp can trigger the polymerization process when interacting with the C-termini of other antibodies such as IgG.

1.4.4. Plant-based Vaccines for livestock diseases in Vietnam

In Vietnam, there have been no reported studies on the development of plant-based vaccines for PEDV prevention.

Chapter 2. MATERIALS AND METHODS

2.1. Materials

2.2.1. Strain of microorganism, cell, plasmid, and gene sequence

The list of microbial strains, cells, and plasmids used in the thesis is presented in Table 2.1**.**

2.1.2. Primers

The list of primers designed and used in the thesis is presented in Table 2.2.

2.1.3. Plant materials

Nicotiana benthamiana plants hydroponically cultivated at 6-8 weeks old.

2.1.4. Animal materials

Female white mice aged 4-5 weeks, pregnant sows, and piglets aged 4-5 weeks.

2.1.5. Chemicals

2.1.6. Equipment

2.2. Methods

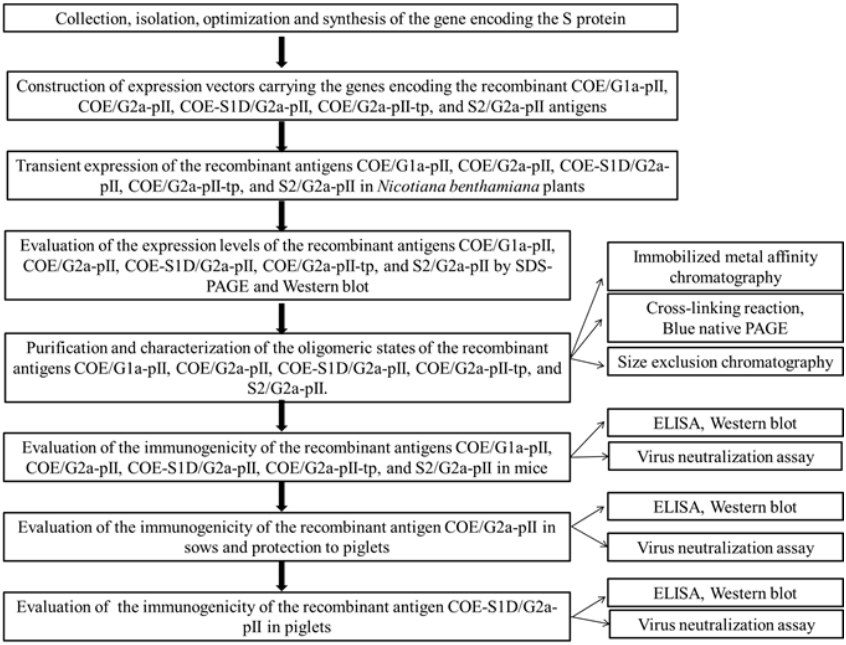

Figure 2.1. Diagram summarizing the research methods used in the thesis

Chapter 3. RESULTS

3.1. Production and evaluation of the immunogenicity of the recombinant COE/G1a-pII antigen from the PEDV strain belonging to the G1a group

3.1.1. Contruction of the expression vector carrying the gene encoding the COE/G1a-pII antigen and creation of an A. tumefaciens strain carrying the corresponding vector

The results of selecting bacterial strains through colony-PCR and digestion plasmids with the *Nco*I indicated the successful construction of the pCB301-COE/G1a-pII expression vector and the creation of the corresponding *A. tumefaciens* strain.

3.1.2. Transient expression of the COE/G1a-pII antigen in N. benthamiana leaves

Six days after agroinfiltration, the tobacco leaves were harvested. The accumulation level of the COE/G1a-pII antigen in the tobacco leaves reached approximately 234 mg/kg of fresh leaves, accounting for about 4% of the total soluble protein (TSP).

3.1.3. Purification and characterization of the oligomeric state of the COE/G1a-pII antigen

Based on the results of total protein measurement using the Bradford assay and quantitative analysis via Western blot, the COE/G1a-pII antigen was determined to have a purity level of approximately 85%. After purification via IMAC, the recovery efficiency of the COE/G1a-pII antigen from the plant extract was approximately 35%.

The results of SDS-PAGE electrophoresis, Coomassie blue staining, and Western blot indicated the presence of a band at approximately 100 kDa,

which corresponds to the size of the COE/G1a antigen in trimeric form in the presence of BS3. This demonstrates that the COE/G1a trimeric antigen was successfully formed upon the conjugation of COE/G1a with the pII motif.

3.1.4. Evaluation of the immunogenicity of the recombinant COE/G1a-pII antigen in mice

Regarding the PEDV-specific IgG antibodies, the crude plant extract containing the COE/G1a-pII antigen showed a level of stimulation in the production of PEDV DR13-specific IgG antibodies similar to that of the commercial CTC Vacc PED vaccine (from South Korea) after the third injection. The plant extract containing the COE/G1a-pII antigen also stimulated the production of specific IgA and IgM antibodies against PEDV.

In terms of the neutralizing antibodies against PEDV, there was no statistically significant difference between the neutralizing antibody titers in the sera of mice vaccinated with crude plant extract containing COE/G1a-pII (G3) and those vaccinated with the commercial CTC Vacc PED (South Korea) containing the PEDV DR13 strain (G2), with a p-value of 0.187. The sera from both the group of mice vaccinated with crude plant extract containing COE/G1a-pII (G3) and the commercial vaccine (G2) exhibited the ability to neutralize PEDV, with neutralizing antibody titers reaching 57.6 and 61.86, respectively. The inhibitory effect on PEDV by neutralizing antibodies in the control group of mice (G1) was very low, with neutralizing antibody titers close to 1 (Figure 3.8). Therefore, these results demonstrate that the plant extract containing the COE/G1a-pII antigen stimulated the production of neutralizing antibodies with similar efficacy to the commercial CTC Vacc PED (South Korea) vaccine against the PEDV DR13 strain.

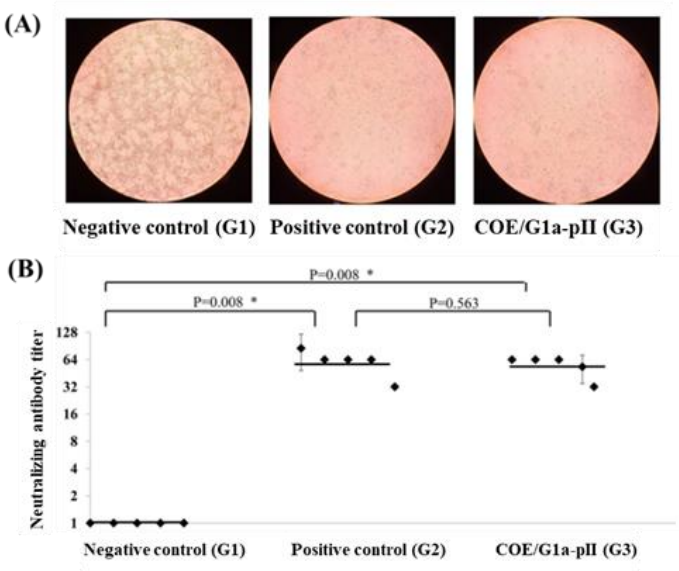

Figure 3.8. Evaluation of the induction of neutralizing antibodies against PEDV in mouse groups *via* virus neutralization assays on cells

A. Microscopic images of observed cytopathic effects. B. Neutralizing antibody titers in the vaccinated mouse groups. $p<0.05$: statistically significant difference.

3.2. Production and evaluation of the immunogenicity of the recombinant COE/G2a-pII antigen from the PEDV strain belonging to the G2a group causing disease in Vietnam

3.2.1. Collection, selection, and isolation the gene encoding the S protein from the PEDV strain causing disease in Vietnam

The gene sequence encoding the S protein of the highly virulent NAVET/PEDV/PS6/2010 strain was selected. This strain was successfully isolated and cultured in Vietnam. The results of constructing a phylogenetic tree based on the gene sequence encoding the S protein indicated that the highly virulent NAVET/PEDV/PS6/2010 strain belongs to the G2a genogroup.

3.2.2. Contruction of the expression vector carrying the gene encoding the COE/G2a-pII antigen and creation of an A. tumefaciens strain carrying the corresponding vector

The results of selecting bacterial strains through colony-PCR and digestion plasmids with the *Nco*I indicated the successful construction of the pCB301-COE/G2a-pII expression vector and the creation of the corresponding *A. tumefaciens* strain.

3.2.3. Transient expression of the COE/G2a-pII antigen in N. benthamiana leaves

The COE/G2a-pII antigen accumulates in tobacco leaves at approximately 118 mg/kg of fresh leaves, accounting for 2.01% of the total soluble protein (TSP). The expression of the COE/G2a antigen recombination in *N. benthamiana* leaves was detected using porcine serum from pigs vaccinated with the Corning vaccine containing the inactivated PEDV AJ1102 strain (Wuhan Keqian Biology). These results demonstrated that the COE/G2a-pII protein produced in *N. benthamiana* leaves possesses antigenic properties similar to the COE antigen of natural PEDV.

3.2.4. Purification and characterization of the oligomeric state of the COE/G2a-pII antigen

3.2.4.1. Purification of COE/G2a-pII antigen by IMAC

The washing buffer supplemented with 10 mM imidazole was suitable for the purification of the COE/G2a-pII antigen. Based on the results of total protein measurement using the Bradford assay and quantitative analysis via Western blot, the COE/G2a-pII antigen was determined to have a purity level of approximately 82%. After IMAC purification, the recovery efficiency of the COE/G2a-pII antigen from the plant extract was approximately 25%.

3.2.4.2. Purification and characterization of the oligomeric state of the COE/G2a-pII antigen using Size-Exclusion Chromatography (SEC) and cross-linking reaction

The results of the oligomeric state analysis of the COE/G2a-pII antigen using SEC and the BS3 cross-linking reaction confirm that the COE/G2a-pII antigen naturally exists in an oligomeric form with a molecular weight ranging from approximately 158-440 kDa. The differences in the natural molecular weight between the COE/G1a-pII and COE/G2a-pII proteins can be attributed to variances in the amino acid sequences of the two proteins, leading to differences in the number of glycosylation sites on the proteins. These differences might affect the separation of the proteins on both gel and SEC columns.

3.2.5. Evaluation of the immunogenicity of the recombinant COE/G2a-pII antigen in pigs

3.2.5.1. The immune response of pregnant sows vaccinated with COE/G2a-pII antigen

Regarding the PEDV-specific IgG antibodies, ELISA results indicated that the COE/G2a-pII antigen stimulated the production of specific IgG antibodies against PEDV in pregnant sows on days 35 and 50 after vaccination. No PEDV-specific IgG antibodies were detected in pregnant sows that received PBS on days 35 and 50 after vaccination. Regarding the PEDV-specific IgA antibodies, ELISA results showed the presence of PEDV-specific IgA antibodies in the serum of pregnant sows vaccinated with the COE/G2a-pII antigen on days 35 and 50. Specific IgA antibodies against PEDV were not detected in the serum of pregnant sows that received PBS on days 35 and 50 after vaccination.

Regarding the neutralizing antibodies against PEDV, the COE/G2a-pII antigen stimulated the production of neutralizing antibodies against PEDV in pregnant sows after vaccination, with an average neutralizing antibody titer of 32 on day 35. No neutralizing antibodies against PEDV were

detected in the serum of pregnant sows that received PBS on days 35 and 50 after vaccination (Figure 3.19).

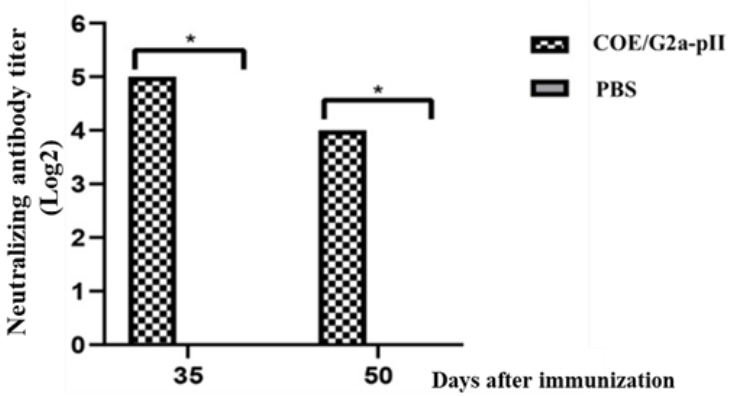

Figure 3.19. Neutralizing antibodies against PEDV in the serum of pregnant sows after vaccination

Regarding the IFN-γ cytokine response, ELISA results showed that the serum of pregnant sows vaccinated with the COE/G2a-pII antigen exhibited a high IFN-γ response, reaching a concentration of 932.3 ± 145.2 pg/ml on day 35 after vaccination. Notably, this IFN-γ level increased two-fold (1796.1 ± 8.6 pg/ml) in these sows on day 50 after immunization. IFN-γ was not detected in the serum of control sows that received PBS on days 35 and 50 after immunization. The data indicate that the COE/G2a-pII antigen stimulated the production of both humoral and cellular immune responses in pregnant sows.

3.2.5.2. The humoral immune response and cytokine response in piglets born from vaccinated pregnant sows

The ELISA results showed the presence of PEDV-specific IgG antibodies in the serum of 5 day-old piglets, both before and after the virulent challenge. The PEDV-specific IgG antibody responses were not detected in

piglets born from sows that received PBS on day 10 post-virulent challenge. ELISA results also indicated the presence of COE-specific IgA antibodies in the serum of piglets before and after the virulent challenge. The COE-specific IgA antibodies were not detected in the serum of sows received PBS before and on day 10 post-virulent challenge.

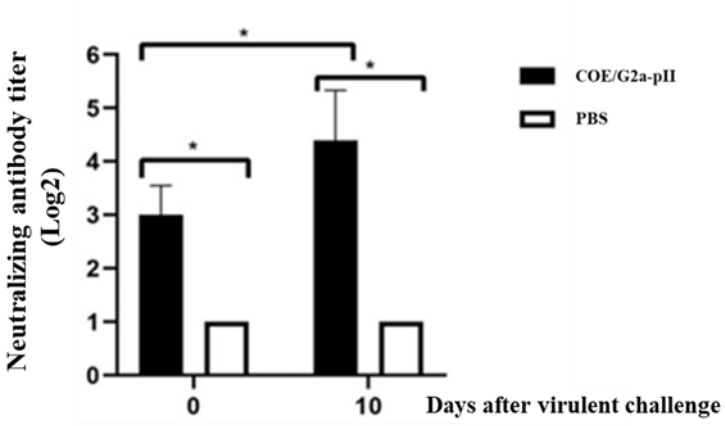

Figure 3.22. Maternal antibody neutralizing response against PEDV in the sera of piglets born from sows vaccinated before and after virus challenge

Regarding the neutralizing antibody immune responses against PEDV, the neutralizing antibody titer against PEDV detected in piglets on the 10th day after the virulent challenge was two-fold higher than before the virulent challenge, with a titer reaching 16. Neutralizing antibodies against PEDV were not detected in the serum of piglets born from sows vaccinated with PBS before and 10 days after the virulent challenge (Figure 3.22).

The ELISA results showed a significant increase in the concentration of IFN-γ in piglets born from sows immunized with the COE/G2a-pII antigen on the 10th day after the virulent challenge. In contrast, the IFN-γ cytokine response was not detected in piglets born from sows that received PBS before and 10 days after the virulent challenge. These data indicated the

presence of passive immunity transfer of both PEDV-specific IgG and IgA antibodies with neutralizing capabilities and the IFN-γ response from sows to piglets through colostrum/early milk.

3.2.5.3. The protective immune response against the highly virulent PEDV G2a strain in piglets

All piglets born from the sow vaccinated with COE G2a-pII antigen returned to the fecal score of 0 on day 6. In contrast, piglets born from the sow vaccinated with PBS had a fecal score of 3 from day 2 onwards. Before the virulent challenge, there was no significant difference in the body weight of the two groups of piglets. Ten days after the virulent challenge, the body weight of piglets born from the COE/G2a-pII vaccinated sow had doubled. Conversely, the body weight of two piglets born from the control sow decreased significantly after the virulent challenge compared to before the virulent challenge.

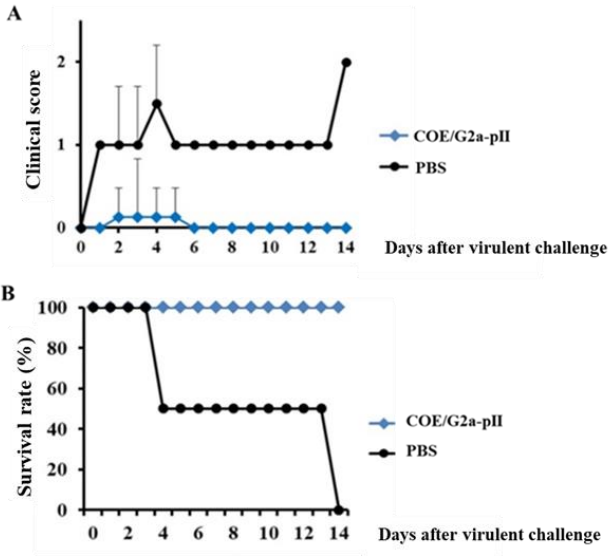

Figure 3.25. Clinical scores (A) and survival rate (B) of piglets born from sows vaccinated before and after virus challene

All piglets born from the sow vaccinated with COE/G2a-pII antigen had normal clinical scores around 0 and a 100% survival rate after the virulent challenge (Figure 3.25A, B). In contrast, clinical characteristics of piglets born from the sow vaccinated with PBS were around 1 (lethargy, weakness, vomiting, and huddling) starting from day 2, and the clinical score for this group increased to 1.5 on day 4 (Figure 3.25A). All piglets in the negative control group died after the virulent challenge (Figure 3.25B).

3.3. Production and evaluation of the immunogenicity of the recombinant COE-S1D/G2a-pII, COE/G2a-pII-tp and S2/G2a-pII antigens from the PEDV strain belonging to the G2a group causing disease in Vietnam

3.3.1. Contruction of the expression vector carrying the genes encoding the COE-S1D/G2a-pII, COE/G2a-pII-tp, S2/G2a-pII antigens and creation of A. tumefaciens strains carrying the corresponding vectors

The results of selecting bacterial strains through colony-PCR and digestion plasmids with the *Nco*I indicated the successful construction of the pCB301-COE-S1D/G2a-pII, pCB301-COE/G2a-pII-tp, pCB301-S2/G2a-pII expression vectors and the creation of the corresponding *A. tumefaciens* strains.

3.3.2. Transient expression of the COE-S1D/G2a-pII, COE-pII-tp và S2/G2a-pII antigens in N. benthamiana leaves

The results of quantitative Western blot analysis showed that the antigens COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII accumulated in tobacco leaves at levels of 115 mg/kg fresh leaves, accounting for 1.95% of the total soluble protein (TSP); 590 mg/kg fresh leaves, accounting for 5.62% of TSP; and 86.7 mg/kg fresh leaves, accounting for approximately 1.47% of TSP, respectively. The expression of COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII antigens in *N. benthamiana* leaves was

also detected using serum from pigs vaccinated with the Corning vaccine containing inactivated PEDV AJ1102 strain (Wuhan Keqian Biology). These results indicated that the COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII proteins produced in *N. benthamiana* leaves exhibit antigenic activity similar to the antigens of natural PEDV.

3.3.3. Purification and characterization of the oligomeric state of the COE-S1D/G2a-pII, COE/G2a-pII-tp, S2/G2a-pII antigens

After purification by IMAC, the COE-S1D/G2a-pII antigen achieved approximately 80% purity, with a recovery yield of approximately 27% from the plant extract. The natural form of the COE-S1D/G2a-pII antigen exists as an oligomer with a molecular weight ranging from 440 kDa to 669 kDa. After purification by IMAC, the COE/G2a-pII-tp antigen reached approximately 82% purity, with a recovery yield of approximately 20% from the plant extract. The natural form of the COE/G2a-pII-tp antigen exists as an oligomer with a molecular weight ranging from 158 kDa to 669 kDa. After purification by IMAC, the S2/G2a-pII antigen achieved approximately 81% purity, with a recovery yield of approximately 22% from the plant extract. The natural form of the S2/G2a-pII antigen exists as an oligomer with a molecular weight ranging from 440 kDa to 669 kDa.

3.3.4. Evaluation of the immunogenicity of the recombinant COE-S1D/G2a-pII, COE/G2a-pII-tp và S2/G2a-pII antigens in mice

The immune responses of the recombinant antigens COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII were compared to that of the COE/G2a-pII antigen in mice.

In terms of the PEDV-specific IgG antibody response, both COE-S1D/G2a-pII and COE/G2a-pII-tp antigens stimulated a stronger production of PEDV-specific IgG antibodies compared to the COE/G2a-pII antigen. All

three antigens, COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII, were found to have the ability to stimulate the production of PEDV-specific IgG antibodies at equivalent levels.

Regarding the PEDV-specific IgA antibody response, the antigens COE-S1D/G2a-pII and COE/G2a-pII-tp have stimulated the production of PEDV-specific IgA antibodies more effectively than the COE/G2a-pII antigen. The COE/G2a-pII-tp antigen induced stronger production of the PEDV-specific IgA antibodies compared to the other antigens. The S2/G2a-pII antigen elicited a similar PEDV-specific IgA antibody response as the COE/G2a-pII antigen.

Regarding the PEDV-specific IgM antibody response, the antigens COE-S1D/G2a-pII and COE/G2a-pII-tp stimulated the production of th PEDV-specific IgM antibodies against PEDV more effectively than the COE/G2a-pII antigen. In contrast, the S2/G2a-pII antigen induced a lower PEDV-IgM antibody response compared to the COE/G2a-pII antigen.

Regarding the neutralizing antibody response against PEDV, the antigens COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII stimulated the production of neutralizing antibodies against PEDV that were equivalent to the COE/G2a-pII antigen.

Regarding the IFN-γ cytokine response, the COE/G2a-pII antigen stimulated a stronger production of IFN-γ response compared to the antigens COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII.

3.3.5. Evaluation of the immunogenicity of the recombinant COE-S1D/G2a-pII antigen in pigs

The immunogenicity of antigen COE-S1D/G2a-pII was compared to that of antigen COE/G2a-pII in 4-5-week-old piglets.

Experiment 1: Comparison of the immunogenicity of antigens COE-S1D/G2a-pII and COE/G2a-pII (dose: 12.5 µg) in piglets; evaluating the impact of adjuvants Montanide ISA™ 201 (SEPPIC) and Emulsigen®-D (MVP) on the immunogenicity of antigen COE-S1D/G2a-pII.

Regarding the PEDV-specific IgG antibody response, the COE-S1D/G2a-pII antigen elicited a PEDV-IgG antibody response equivalent to the COE/G2a-pII antigen when mixed with the adjuvant Montanide ISA™ 201 in piglets. When mixed with the COE-S1D/G2a-pII antigen, the adjuvant Emulsigen®-D was more effective in stimulating the production of PEDV-specific IgG antibodies compared to the adjuvant Montanide ISA™ 201.
Regarding the PEDV-specific IgA antibody response, the COE-S1D/G2a-pII antigen induced a stronger PEDV-IgA antibody response compared to the COE/G2a-pII antigen when combined with the adjuvant Montanide ISA™ 201 in piglets. When mixed with the COE-S1D/G2a-pII antigen, the adjuvant Emulsigen®-D demonstrated better efficacy than Montanide ISA™ 201 in stimulating the production of the PEDV-specific IgA antibody response.

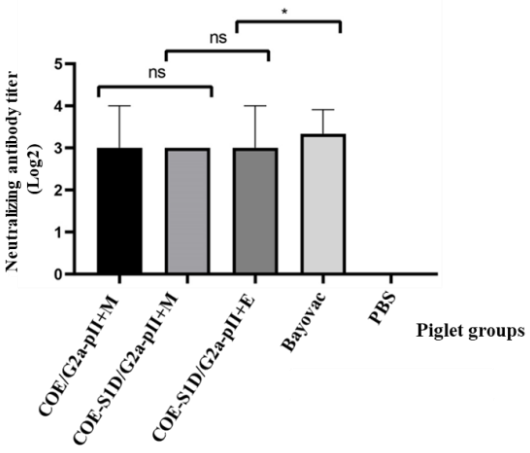

Figure 3.49. Evaluation of neutralizing antibody response against PEDV of the serum of piglet groups using a virus neutralization assay

In terms of the neutralizing antibody response against PEDV, the COE-S1D/G2a-pII antigen stimulated the production of neutralizing antibodies against PEDV equivalent to the COE/G2a-pII antigen in piglets. When combined with the COE-S1D/G2a-pII antigen, both the adjuvants Montanide ISA™ 201 and Emulsigen®-D have induced the production of neutralizing antibodies against PEDV at equivalent levels in piglets (Figure 3.49).

Regarding the IFN-γ response, the COE-S1D/G2a-pII antigen elicited an IFN-γ response equivalent to the COE/G2a-pII antigen when mixed with the adjuvant Montanide ISA™ 201 in piglets. When combined with the COE-S1D/G2a-pII antigen, the Emulsigen®-D adjuvant promoted stronger production of IFN-γ response than the Montanide ISA™ 201 adjuvant.

Experiment 2: Comparing the immunogenicity of the antigen COE-S1D-G2a-pII (dose: 50 µg) with a commercial vaccine in piglets

The immunogenicity of the COE-S1D/G2a-pII antigen (dose: 50 µg) was compared to the immunogenicity of the Corning vaccine, which contains the inactivated PEDV strain AJ1102 (Wuhan Keqian Biology) belonging to group G2b in piglets. Regarding the PEDV-specific IgG antibody response, ELISA results showed that after the second vaccination, the PEDV-specific IgG antibody response in the group of piglets vaccinated with COE-S1D/G2a-pII mixed with the adjuvant Emulsigen®-D was higher than that in the group of piglets vaccinated with the commercial Corning vaccine ($p<0.05$). This was reflected in the average OD450 values of 3.8 and 2.087 for the two groups, respectively. The PEDV-specific IgG antibody response was not detected in the group of piglets vaccinated with PBS mixed with Emulsigen®-D after the first and second immunization.

Regarding the PEDV-specific IgA antibody response, ELISA results showed that after the second immunization, there was an increase in the PEDV-specific IgA antibody response in the group of piglets vaccinated

with COE-S1D/G2a-pII along with the adjuvant Emulsigen®-D, with an OD450 value of 1.57. In contrast, there was a slight decrease in the PEDV-specific IgA antibody response in the group of piglets vaccinated with the commercial Corning vaccine. However, there was no statistically significant difference in the PEDV-specific IgA antibody response between these two groups, with a p-value greater than 0.05. The PEDV-specific IgA antibody response was not detected in the group of piglets vaccinated with PBS mixed with Emulsigen®-D.

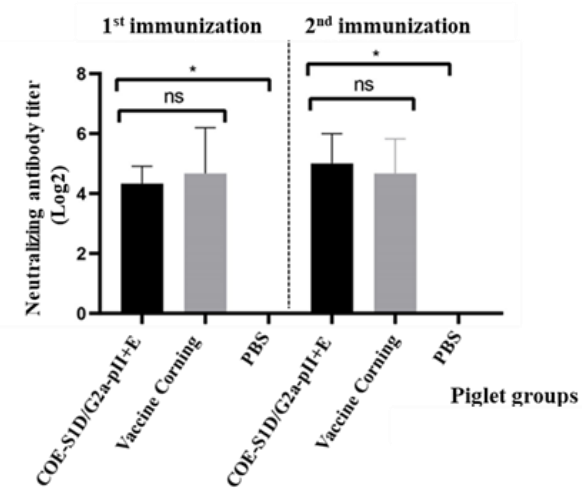

Figure 3.55. Evaluation of neutralizing antibody response against PEDV in piglets using virus neutralization assay.
E: Emulsigen®-D (MVP)

In terms of the neutralizing antibody response against PEDV, after two immunizations, the average neutralizing antibody titer in the group of piglets vaccinated with COE-S1D/G2a-pII along with the adjuvant Emulsigen®-D was 32, whereas the average neutralizing antibody titer in the group of piglets vaccinated with the commercial Corning vaccine was 25.45. However, there was no statistically significant difference in the production of neutralizing antibodies against PEDV between these two

groups, with a p-value greater than 0.05. Neutralizing antibodies against PEDV were not detected in the group of piglets vaccinated with PBS mixed with Emulsigen®-D (Figure 3.55).

Chapter 4. DISCUSSION

4.1. Expression levels and recoversion capability of recombinant antigens from *N. benthamiana* leaves

4.2. Immunogenicity of COE/G1a-pII antigen in mice

4.3. Immunogenicity of COE/G2a pII antigen in sows and protective ability against highly virulent PEDV G2a strain in piglets

4.4. Comparison of immunogenicity between recombinant antigens COE-S1D/G2a-pII, COE/G2a-pII-tp, and S2/G2a-pII with COE/G2a-pII antigen in mice

4.5. Comparison of immunogenicity between COE-S1D/G2a-pII and COE/G2a-pII antigens in piglets

CONCLUSION AND RECOMMENDATIONS

CONCLUSION

1. The antigen COE/G1a-pII from the PEDV strain DR13 belonging to the G1a subgroup was successfully expressed in *N. benthamiana* leaves at a level of 234 mg/kg of fresh leaves. Leaf extract containing the COE/G1a-pII antigen stimulated the production of specific PEDV-IgG, IgA, and IgM antibodies that had the ability to neutralize PEDV with high neutralizing antibody titers in mice. The ability to induce neutralizing antibodies against PEDV of leaf extract containing the COE/G1a-pII antigen was equivalent to the commercial CTC Vacc PED vaccine (from South Korea) in mice.
2. The COE/G2a-pII antigen from the highly virulent PEDV strain NAVET/PEDV/PS6/2010 belonging to the G2a subgroup was successfully expressed in *N. benthamiana* leaves at a level of 118 mg/kg of fresh leaves. The COE/G2a-pII antigen stimulated the production of PEDV-specific IgG and IgA antibodies, that had the ability to neutralize PEDV, and high levels of IFN-γ in sows after two immunizations (100 µg/dose). Following the virulent challenge experiment using the highly virulent PEDV G2a strain, 100% of piglets born from the sow vaccinated with the COE/G2a-pII antigen survived, exhibited normal clinical signs, and showed increased body weight.
3. The antigens COE/G2a-pII-tp, COE-S1D/G2a-pII, and S2/G2a-pII from the highly virulent PEDV strain NAVET/PEDV/PS6/2010 belonging to the G2a subgroup were successfully expressed in *N. benthamiana* leaves, with at levels of 590, 115, and 86.7 mg/kg of fresh leaves, respectively. The COE/G2a-pII-tp antigen induced the strongest production of PEDV-specific IgA antibodies among all the antigens tested in mice. The COE-S1D/G2a-pII antigen stimulated the production of PEDV-specific IgA antibodies better than the COE/G2a-pII antigen in piglets. When combined with the COE-S1D/G2a-pII antigen, the Emulsigen®-D (MVP) adjuvant was more effective than the Montanide

ISA™ 201 (SEPPIC) adjuvant in stimulating the production of PEDV-specific IgG and IgA antibodies and IFN-γ response in piglets. The COE-S1D/G2a-pII antigen (50 µg/dose) combined with Emulsigen®-D demonstrated equivalent efficacy to the commercial Corning vaccine (Wuhan Keqian Biology) in inducing neutralizing antibodies against PEDV in piglets. The production of PEDV-specific IgG antibodies in piglets receiving two doses of the COE-S1D/G2a-pII antigen (50 µg/dose) with Emulsigen®-D adjuvant was higher than that in piglets vaccinated with the commercial Corning vaccine.

RECOMMENDATIONS:

1. Development and optimization of the production process of the COE/G2a-pII antigen in *N. benthamiana* plants and assession its ability to stimulate protective immunity in a large number of piglets born from sows vaccinated with the COE/G2a-pII antigen against the circulating PEDV strain belonging to the G2 subgroup in Vietnam.
2. Immunization of the COE-S1D/G2a-pII and COE/G2a-pII-tp antigens in sows and evaluation of the protective capacity of piglets born from vaccinated sows following the challenge experiment to the locally circulating PEDV strain belonging to the G2 subgroup in Vietnam.

LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

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2. **Ho Thi Thuong**, Trinh Thai Vy, Nguyen Thi Tra, Hoang Thi Thu Hang, Pham Bich Ngoc, Chu Hoang Ha, 2021, Construction and optimization of transient expression of the COE trimer protein from the Porcine epidemic diarrhea virus strain causing disease in Vietnam in *Nicotiana benthamiana*, *Vietnam Biotechnology Conference Proceedings*, pp. 2-8. ISBN 978-604-9987-88-5.
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4. **Ho Thi Thuong**, Trinh Thai Vy, Hoang Thi Thu Hang, Pham Bich Ngoc, Chu Hoang Ha, 2022, Transient expression and purification of S2 protein from Porcine epidemic diarrhea virus in plants, *Vietnam Journal of Biotechnology*, 20 (4), tr. 1-11. doi: 10.15625/1811-4989/1750.
5. **Ho Thi Thuong**, Trinh Thai Vy, Nguyen Thi Thu Hien, Lê Thi Tra My, Pham Đinh Minh, Pham Bich Ngoc, Hoang Thi Thu Hang, Chu Hoang Ha, 2023, The immunogenicity of the S2 antigen from the Porcine epidemic diarrhea virus (PEDV) fused with the GCN4pII motif in mice, *TNU Journal of Science and Technology*, 228(13), pp. 298 – 305. doi: https://doi.org/10.34238/tnu-jst.8349.