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***Research Paper***

# Generation of recombinant Bacillus subtilis expressing Porcine Epidemic Diarrhea Virus (PEDV) S1 protein in vegetative cell

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## Abstract

Porcine epidemic diarrhea virus (PEDV) is a mucosal (gut surface) pathogen that causes severe diarrhea in piglets; thus, a vaccine capable of inducing gut-mucosal immune response is crucial for controlling PEDV infection. Bacillus subtilis has been considered a choice for vaccine delivery to the gut mucosa. In this study, we aimed to generate recombinant B. subtilis that can produce PEDV S1 protein in vegetative cell. Two promoters, PrrnO and PgsiB-PsecA, were selected for an early and high yield expression of PEDV S1 gene in B. subtilis vegetative cell and germinating spore. Promoters, PrrnO and PgsiB-PsecA, were linked to the 5' end of the fusion gene pgsA-PEDVS1 and the fusion genes were then inserted into plasmid pDG1662. Recombinant B. subtilis strains were generated by integrating the fusion genes into B. subtilis 168 chromosome via double crossover homologous recombination. PCR amplification and amylase activity assay confirmed integration of the fusion genes into B. subtilis chromosome at amyE locus. Expression of the pgsA-PEDVS1 in B. subtilis vegetative cells germinating from spores was then studied at 2, 4, 8 and 16 hours of culture. Tested by western blot analysis, although only cleaved products of PgsA-PEDVS1 protein were observed, expression levels of pgsA-PEDVS1 under the control of both promoters were comparable at all time points. Importantly, PgsA-PEDVS1 protein could be detected as early as 2 hours after spore inoculation in LB medium. This study suggests that both PgsiB-PsecA and PrrnO promoters can be used for efficient production of PEDV S1 in germinating spore and vegetative cell and may be applicable for expression of other heterologous genes in B. subtilis vegetative cell.

***Keywords***: *Bacillus Subtilis, PEDV, Spike Protein, Mucosal Vaccine*

## 1. Introduction

Porcine epidemic diarrhea virus (PEDV) is an enveloped, positive-sense, single-stranded RNA virus [1]. It is the causative agent of porcine epidemic diarrhea (PED), a highly contagious, enteric disease of swine [2]. PEDV infection causes vomiting, and dehydration in piglets, resulting in a high mortality (sometimes 100% loss) of newborn piglets, thus causing great economic loss in swine industry in Asian and north America countries [3].

PED is considered a mucosal disease, by which PEDV infections are characterized by acute destruction of intestinal villous enterocytes and villous atrophy within the jejunum and ileum [4]. Villous enterocytes in the small and large intestine are the primary as well as the main sites for PEDV infection and replication [5]. Thus, a mucosal vaccine capable of inducing mucosal immune response, particularly in the gut mucosa, is crucial for controlling PEDV infection.

The vaccine targeted to mucosal tissues could be achieved by various strategies, one of which is the use of *Bacillus subtilis* for vaccine production and delivery. *B. subtilis* offers several advantages to be used as a vaccine delivery vehicle including (i) it is normal flora that exists in intestine, (ii) it is recognized as generally recognized as safe (GRAS) organism, (iii) methods for genetic manipulation are well established (iv) it has high density cell growth; thus, the production cost is low, (v) it can be given orally, thereby decreasing the need of needle use, and (vi) it can produce spore when the condition is not suitable for growth [6].

Often, when *B. subtilis* is used for vaccine delivery to gut mucosa, spore form is a choice because it can tolerate acidic condition in the gut. However, it has been reported that spore delivered to the animal intestine can germinate into vegetative cell [7]. We therefore though that *B. subtilis* capable of expressing vaccine antigen in germinating spore and vegetative cell should enhance the immunogenicity of *B. subtilis* spore-based vaccine. Here, we first study the expression of vaccine antigen derived from PEDV protein in germinating spore and vegetative cells.

PEDV spike (S) protein, which plays an important role in virus attachment to its receptor on host cell, is the main target for vaccine development [8]. The S protein of PEDV is a class I membrane glycoprotein consisting of two subunits: N-terminal S1 and C-terminal S2 [9]. Vaccine developed based on PEDV S1 subunit has been shown to stimulate production of neutralizing antibodies and vaccine-induced neutralizing antibodies can be maternally transferred to suckling piglets and protected piglets from PEDV infection [10]. In this study, we therefore used S1 subunit as a vaccine antigen and to be expressed by *B. subtilis* vegetative cell.

When expressed in *B. subtilis* vegetative cell, PEDV S1 was aimed for surface-display; thus, a carrier protein is needed. In our study, poly gamma-glutamate synthetase complex (PgsA) [11] of *B. subtilis*, which consist mainly of hydrophobic and positive charged amino acid residues, was used as a carrier protein for presenting and anchoring PEDV S1 to the cell membrane. To

obtain highest yield of PEDVS1 expression in vegetative cells, we studied expression efficiency of 2 different promoters, P*rrnO* [12] and P*gsiB*-P*secA* [13], which have been shown to highly express transgene in germinating spore and vegetative cell, respectively. Using western blot analysis, only degraded forms of PEDV S1 were observed and both promoters show comparable ability to express PEDV S1.

## 2. Materials and methods

### 2.1. DNA extraction from *B. subtilis* 168

Genomic DNA of *B. subtilis* was extracted from *B. subtilis* strain 168 cultured in LB medium (10 g $L^{-1}$ of tryptone, 5 g $L^{-1}$ of yeast extract and 5 g $L^{-1}$ of Sodium chloride), using TIANamp genomic DNA kit (TIANGEN), following the manufacturer's instruction. DNA samples are stored at -20°C until used.

### 2.2. PCR Amplification the PEDVS1 gene constructs

Four gene fragments including Linker-PEDVS1, P*rrnO*, P*gsiB*-P*secA* and pgsA were generated by PCR amplification. Primers used for PCR amplification are listed in **Table 1**. Linker-PEDVS1 was amplified using cDNA of PEDV as a template with primers Linker-XmaI-PEDVS1-F, V5-AgeI-PEDVS1-R1, CotB_ter/1-V5-R2 and E-H-CotB_ter/2-R3. DNA fragments of P*rrnO* and *pgsA* were amplified from chromosomal DNA of *B. subtilis* 168. P*rrnO* fragment was amplified with primers *BamH*I-PrrnO-F and *Not*I-PrrnO-RBS-R. Gene fragment of *pgsA* was amplified with primers pSecA-NotI-pgsA-F and Linker-KpnI-pgsA-R. Promoter fragment of P*gsiB*-P*secA* was amplified using primers BamHI-PgsiB-F and NotI-PsecA-R and template plasmid pRB373.P*gsiB*-P*secA*-GFP, which was kindly provided by Dr. Teva Phanaksri, Thammasart University. PCR reactions were performed in a 50-µl reaction using KAPA HIFI DNA Polymerase (KAPA BIOSYSTEMS), following the manufacturer's instruction. The annealing temperatures were adjusted to be optimal for each set of primers. After PCR amplification, PCR products were separated in agarose gel. DNA fragments at the expected size were excised and gel-purified using Gel/PCR DNA Fragments Extraction Kit (TIANGEN), following the manufacturer's instruction.

Two PEDVS1 gene constructs, P*rrnO-pgsA-PEDVS1* and P*gsiB*-P*secA-pgsA-PEDVS1*, were then generated using overlapping PCR. DNA fragment of P*rrnO-pgsA-PEDVS1* was generated from fragments P*rrnO*, pgsA and Linker-PEDVS1 using primers BamHI-PrrnO-F and E-H-CotB-ter/2-R3. For P*gsiB*-P*secA-pgsA*-PEDVS1 construct, DNA fragment was generated from P*gsiB*-P*secA*, pgsA and Linker-PEDVS1 using primers BamHI-PgsiB-F and E-H-CotB-ter/2-R3. The overlapping PCR was performed in a 50-µl reaction using KAPA HIFI DNA Polymerase (KAPA BIOSYSTEMS). PCR products were separated on a 0.8% agarose gel. Expected PCR products were excised and gel-purified.

### 2.3. Generation of recombinant shuttle vectors

Two hundred nanograms of P*gsiB*-P*secA*-pgsA-PEDVS1 and P*rrnO*-pgsA-PEDVS1 were digested with *BamH*I and *Eco*RI, followed by purification using PCR DNA Fragments Extraction Kit (TIANGEN). In parallel, plasmid pDG1662 was digested with the same restriction enzymes and then gel-purified. The gene fragments were ligated into shuttle vectors pDG1662. Competent *E. coli* DH5α cell was transformed with ligation mixture using heat-chock method. The transformed bacteria were plated onto LB agar plate containing 100 ng/µl of Ampicillin and incubated at 37°C overnight (12-16 hours). Rapid size screening was applied for recombinant clone screening. Colonies were picked from master plates and lysed in lysis buffer (5mM EDTA, 10% (w/v) sucrose, 0.25% (w/v) SDS, 100 mM NaOH, 60 mM KCl, and 0.05% (w/v) bromophenol blue), followed by agarose gel electrophoresis. Recombinant clones were selected for plasmid extraction using TIANprep Rapid Mini Plasmid Kit (TIANGEN). Existence of the insert was confirmed by digestion with *Bam*HI and *Eco*RI.

**Table 1:** Oligonucleotide primers used in this study

| Oligonucleotide | Sequence (5'→3') |
|---|---|
| **Promoter** | |
| *BamHI*-PgsiB-F | AAGTGGATCCCGCATTCTTGAAC-GAC |
| *Not*I-PsecA-R | GCGGCCGCTCATCACACGCC-TATTTTAGAG |
| *BamHI*-PrrnO-F | AAGTGGATCCGCATGAC-CATTATGACTAG |
| *Not*I-PrrnO-RBS-R | GCGGCCGCGTCTCACCTCCTT-GTACAGGTTAAGTTCACCGCATCC |
| **pgsA** | |
| pSecA-*Not*I-pgsA-F | ATAGGCGTGTGATGAGCGGCCG-CATGAAAAAA-GAACTGAGCTTTCAT |
| Linker-*Kpn*I-pgsA-R | TCCTCCTCCTTTCGCCGCCGCTTCT CCTCCTCCGGTACCTTTAGAT-TTTAGTTTGTCACT |
| **PEDVS1** | |
| Linker-*Xma*I-PEDVS1-F | GCGGCGGCGAAAGGAGGAG-GACCCGG-GATGAGGTCTTTAATTTACTTC |
| V5-*Age*I-PEDVS1-R1 | TGTGCTGTCCAGTCCCAG-CAGCGGGTTCGGAATCGGTTTTCC |
| CotB_ter/1-V5-R2 | CTTTATCTGATGCTCCTCCATTATC TATTATGTGCTGTCCAGTCCCAG-CAGCGGGTTC |
| E-H-CotB_ter/2-R3 | AGTTGAATTCAA-GCTTCCTTTAACTGAAAAAGGG-CATCACTTTATCTGATGCTCCTC |
| **AmyE** | |
| amyE-F | TCG GTT TGA AAG GAG GAA GC |

### 2.4. Generation of recombinant *B. subtilis*

Competent *B. subtilis* 168 was prepared by growing in medium A (10X Medium A base (10 g $L^{-1}$ of Yeast extract, 2 g $L^{-1}$ of Casamino acids, 50% Glucose), 10X Bacillus salts (0.16 M of $(NH_4)_2SO_4$, 0.04 M of $K_2HPO_4 \cdot 3H_2O$, 0.44 M of $KH_2PO_4$, 0.002 M of $Na_3C_6H_5O_7$, $8.1 \times 10^{-3}$ M of $MgSO_4 \cdot 7H_2O$)). After $OD_{650}$ reached 0.4-0.6, the culture was further incubated for 90 minutes. Then, 0.05 ml of this culture was transferred and incubated with 0.45 ml of pre-warmed Medium B (Medium A, 50 mM of $CaCl_2 \cdot 2H_2O$ and 250 mM of $MgCl_2 \cdot 6H_2O$) at 37°C for 90 minutes, giving rise to natural competent *B. subtilis* 168.

To generate recombinant *B. subtilis* strains, plasmid DNA (1 µg) was added to competent *B. subtilis*. Transformation mixture was incubated at 37°C for 1 hour and plated onto LB agar containing chloramphenicol. The transformation plate was incubated at 37°C overnight (12-16 hours). Colonies growing on the plate were subjected to recombinant clone confirmation.

### 2.5. Confirmation of PEDVS1 insertion into the *B. subtilis* chromosome

Select clones were cultured in LB medium and subjected to genomic DNA extraction using genomic Extraction Kit (TIANGEN), following the manufacturer's instruction. Integration of P*gsi*-P*secA*-pgsA-PEDVS1 and P*rrnO*-pgsA-PEDVS1 into *B. subtilis* chromosome at *amyE* locus was confirmed by PCR amplification and amylase activity assay.

PCR amplification was performed with primers AmyE-F and Linker-KpnI-pgsA-R using DreamTag DNA polymerase (Thermo Scientific), following the manufacturer's instruction. Amylase activity assay was performed by culturing recombinant *B. subtilis* clones in LB agar plate containing 0.1% starch in parallel with wild type *B. subtilis*. After incubation at 37°C overnight, the plate was stained with iodine and clear zone was observed.

### 2.6. *B. subtilis* spore and vegetative cell culture

*B. subtilis* wild type and recombinant strains were streaked on LB agar plate containing Chloramphenicol, and then cultured at 37°C overnight (12-16 hours). Colonies were picked and cultured in 3 ml of Difco sporulation medium (DSM; 8 g $L^{-1}$ of nutrient broth, 0.013 M KCl, 0.002 M $MgSO_4$, 1 M $Ca(NO_3)_2$, 10 mM $MnCl_2$ and 1 mM $FeSO_4$) and cultured at 37°C. When the $OD_{600}$ reached 0.4-0.6, 1 OD of the culture was transferred into 25 ml of DSM and cultured at 37°C for 48 hours with shaking (200 rpm). For growing vegetative cells from spores, 5 OD of spore was inoculated into LB medium and cultured at 37°C. The cultured cells were harvested at time points 2, 4, 8 and 16, hours post-inoculation. The cells were centrifuged at 4,500xg for 5 minutes before subjecting to Western blot.

### 2.7. Western blot

Protein from cultured cells was prepared by adding lysis buffer (100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS,
0.2% bromophenol blue, 40% glycerol) into the cell pellet. The protein samples were incubated at 95°C for 5 minutes, gently vortexed and centrifuged at 12,000 rpm for 3 minutes. Proteins were separated in 8% SDS-polyacrylamide gel electrophoresis (PAGE). For Western blot analysis, proteins in the gel were transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS at 4°C overnight. Mouse anti-V5 antibody in 2% skim milk/PBS was added into the membrane and incubated at room temperature for 3 hours. The membrane was washed 3 times with PBST (0.1% tween in PBS) and then incubated with anti-mouse IgG HRP in 2% skim milk/ PBS for 1 hour 30 minutes at room temperature. After washing 3 times, the membrane was incubated with the chemiluminescence substrate for 5 minutes. The signal was then detected using blot scanner.

## 3. Results and discussion

### 3.1. Design of PEDVS1 constructs

PEDV S1 in this study was designed to be surface-displayed on the vegetative cell; thus, it is linked with a carrier protein PgsA, resulting in a fusion protein termed PgsA-PEDVS1. Two different constructs were designed by conjugating the 5' end of pgsA-PEDVS1 fragment with 2 different promoters, P*rrnO* and P*gsiB*-P*secA*, giving rise to 2 PEDVS1 constructs: (i) P*gsiB*-P*secA*-pgsA-PEDVS1 and (ii) P*rrnO*-pgsA-PEDVS1. Linker sequence is added between *pgsA* and *PEDVS1* gene to separate the 2 proteins. V5 tag is added to the C-terminus of PEDVS1 to facilitate protein detection. Terminator is added next to the V5 tag sequence to help terminate gene transcription **(Fig.1).**

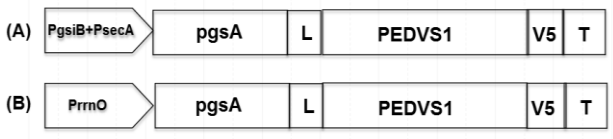


**Fig. 1**: Schematic representation of two fusion genes. (A) P*gsiB*-P*secA*-pgsA-PEDVS1 and (B) P*rrnO*-pgsA-PEDVS1

### 3.2. Generation of PEDVS1 constructs and construction of integrative vector

To generate full-length fragments of 2 fusion genes, P*gsiB*-P*secA*-pgsA-PEDVS1 and P*rrnO*-pgsA-PEDVS1, small fragments were first amplified, followed by overlapping PCR to link all 3 small fragments together and thus result in full-length fragments of fusion genes together with promoters as shown in **Fig. 2**. Overlapping PCR gave products with several bands including the band at around 4 kb, which is the expected product of the fusion genes.

DNA fragments of the fusion genes, P*gsiB*-P*secA*-pgsA-PEDVS1 and P*rrnO*-pgsA-PEDVS1, were gel-purified and digested with restriction enzyme. The fusion genes were then ligated into plasmid pDG1662 between *BamH*I and *EcoR*I**.** Competent *E. coli* DH5α was then transformed with the ligation mixture. The transformed bacterial clones growing on LB agar containing Ampicillin were screened using rapid size screening. The screening result shown that several clones contain insert gene. A few clones from each construct were selected for plasmid extraction. Extracted plasmid were then digested with restriction enzymes, *BamH*I and *EcoR*I, Digestion result showed that recombinant clones number 7, 8, 15, 16 and 18 of pDG1662-pgsiB+psecA-pgsA-PEDVS and recombinant clones number 7, 15 and 18 of pDG1662-PrrnO-pgsA-PEDVS1 contain insert at the expected size, indicating that these pDG1662 clones contain fusion genes. These recombinant plasmids were then used for generation of recombinant *Bacillus subtilis.*

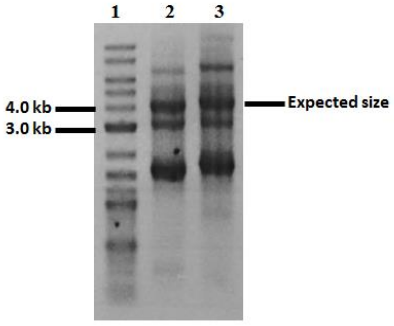


**Fig. 2:** PCR product of P*gsiB*-P*secA*-pgsA-PEDVS1 and P*rrnO*-pgsA-PEDVS1. Lane 1 is Marker. Lane 2 is PCR product of P*gsiB*-P*secA*-pgsA-PEDVS1. Lane 3 is PCR product of P*rrnO*-pgsA-PEDVS1

### 3.3 Generation and confirmation of recombinant *B. subtilis*

To generate recombinant *B. subtilis*, the fusion genes need to be transferred from plasmid pDG1662 into *B. subtilis* chromosome via homologous recombination at *amyE* locus (**Fig. 3**). *B. subtilis* 168 was naturally transformed with plasmid pDG1662.P*gsiB*-P*secA*-pgsA-PEDVS1 and pDG1662.P*rrnO*-pgsA-PEDVS1. Recombinant clones were then selected on the medium containing chloramphenicol.

Integration of the fusion genes at *amyE* locus was then confirmed by PCR and amylase activity assay. PCR was performed using primers that can bind specifically to the *B. subtilis* chromosome and also the fusion gene (AmyE-F and Linker-KpnI-pgsA-R). Expected PCR product at 2 kb was observed in all selected clones **(Fig 4)**, confirming the integration of the fusion genes at *amyE* locus.

For amylase activity assay, recombinant *B. subtilis* clones were cultured in LB agar plate containing 0.1% starch in parallel with wild type *B. subtilis*. After cultured overnight and stained with iodine, clear zone was clearly observed around wild type colonies, while all of the selected recombinant clones did not show clear zone **(Fig 5),** indicating amylase activity disruption. Additionally. Clones number 7.1, 15.2 and 18.1. carrying PrrnO-pgsA-PEDVS1 and clones number 15.6, 16.4 and 18.1 carrying P*gsiB*-P*secA*-pgsA-PEDVS1 were selected for protein expression study

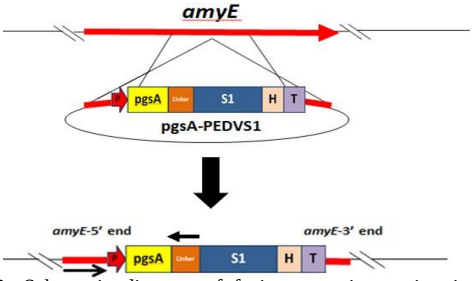


**Fig. 3:** Schematic diagram of fusion gene integration into B. subtilis chromosome via double crossover homologous recombination

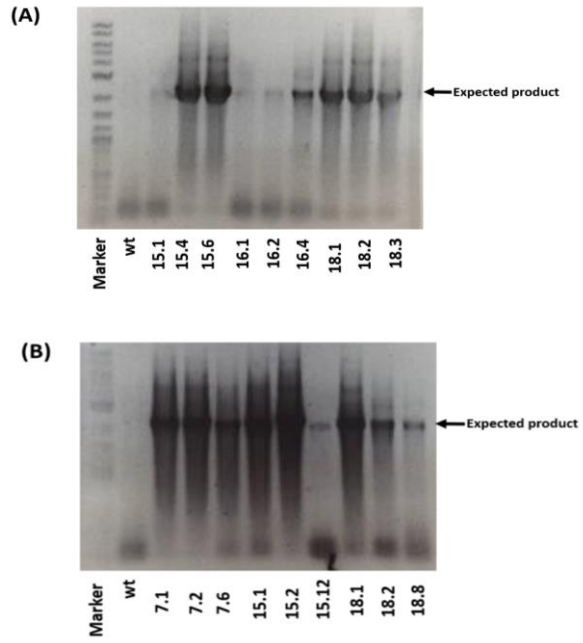


**Fig. 4**. Confirmation of fusion gene integration at *amyE* locus using PCR amplification. PCR was performed using chromosomal DNA from recombinant *B. subtilis* clones transformed with pDG1662.P*rrnO*-pgsA-PEDVS1 (A) and pDG1662.P*gsiB*-P*secA*-pgsA-PEDVS1 (B).

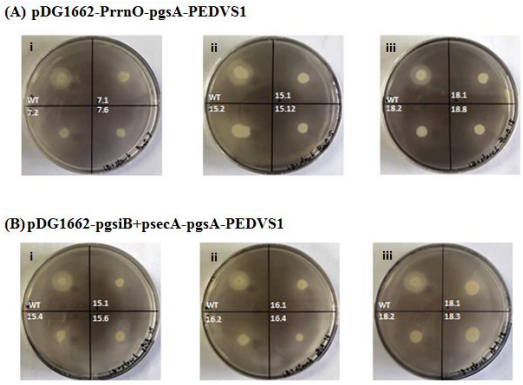


**Fig. 5:** Amylase activity assay. (A) Recombinant *B. subtilis* clones transformed with clone number 7 (i), 15 (ii), and 18 (iii) of pDG1662.P*rrnO*-pgsA-PEDVS1. (B) Recombinant B. subtilis clones transformed with clone number 15 (i), 16 (ii), and 18 (iii) of pDG1662.P*gsiB*-P*secA*-pgsA-PEDVS1. The colonies were cultured with starch and then stained with iodine.

### 3.4. Expression of PEDVS1 protein

To study protein expression of pgsA-PEDVS1 controlled by 2 different promoters, spores of recombinant B. subtilis and wild type were inoculated into LB medium and harvested at time points of 2, 4, 8 and 16 hours. Cultured cells were then collected and the cell pellet were mixed with 2X SDS loading dye, heated and then subjected to SDS-PAGE and Western blot analysis. Anti-V5 antibody was used to detect V5-tagged pgsA-PEDVS1 protein. Using Western blot analysis, no protein at the size of 129 kDa, which is an expected size of pgsA-PEDVS1, was observed, instead, smaller bands were detected at studied time points (**Fig. 6A-D**), These small bands are presumably cleaved products of pgsA-PEDVS1. Based on the small bands detected, pgsA-PEDVS1 expressed by promoters P*gsiB*-P*secA* and P*rrnO* were in comparable levels at all time points (Fig 4.8). This result suggests that both promoters could express pgsA-PEDVS1 as early as the spores started to germinate and continued to express the gene in vegetative cells.

This result suggests that pgsA-PEDVS1 protein may be unstable and can be rapidly degraded by proteases of *B. subtilis*. Expression of PEDVS1 was then further studied in *E. coli* host system. When expressed in *E. coli* BL21 (DE3), PEDVS1 could be observed only in cleaved form, which is corresponding to the result found in *B. subtilis* (**Fig. 6E**),. However, full-length PEDVS1 could be achieved when it was expressed in *E. coli* BL21 (DE3)pLysS (**Fig. 6F**), the cell that provides tighter control of protein expression for expression of toxic proteins. However, all detected protein was in inclusion body. This result suggests that this PEDVS1 protein may be toxic to the cells; thus, small amount of expressed protein can lead to uninduced expression of the protein.

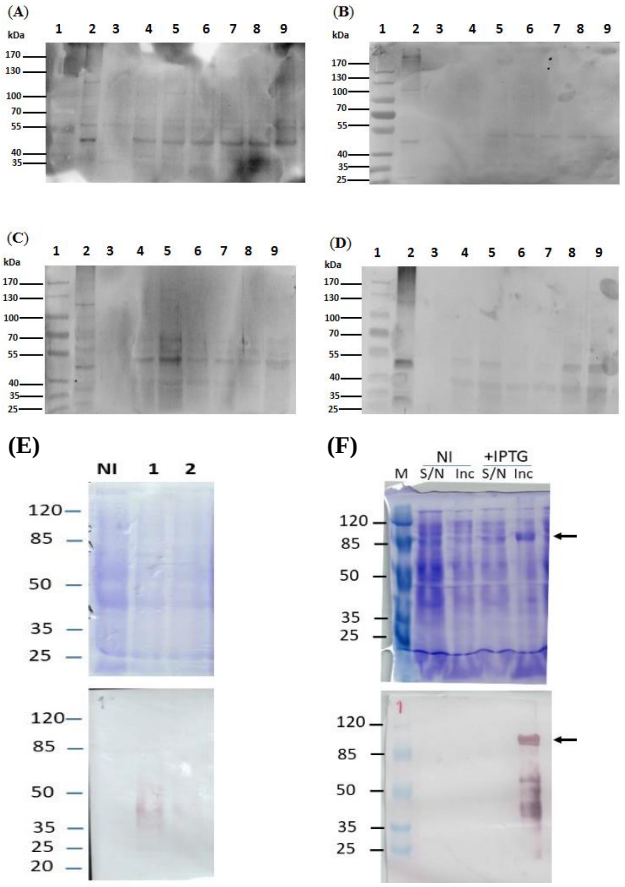


**Fig. 6:** Western blot analysis of PEDVS1 protein. (A) Western blot analysis at 2 hr. (B) Western blot analysis at 4 hr. (C) Western blot analysis at 8 hr. (D) Western blot analysis at 16 hr. Lane 1 Maeker; Lane2 is positive control; Lane 3 is wild- type; Lanes 4-6 recombinant *B. subtilis* by which pgsA-PEDVS1 is expressed by promoter P*rrnO*; Lanes 7-9 recombinant *B. subtilis* by which pgsA-PEDVS1 is expressed by promoter P*gsiB*-P*secA*. (E) Whole cell lysate of PEDVS1 expressed in *E. coli* BL21 (DE3). NI represents no IPTG induction; 1 and 2 indicate clone no 1 and 2 induced with IPTG. induced sample. (F) PEDVS1 expressed in *E. coli* BL21 (DE3)pLysS. NI represents no IPTG induction; +IPTG indicates conditions with IPTG induction; S/N indicates supernatant; Inc indicates inclusion body.

## 4. Conclusions

Mucosal vaccine is considered important in controlling PEDV infection. Here, we developed *B. subtilis*-based PEDV vaccine. PEDVS1, a vaccine antigen derived from PEDV S1 subunit, was fused with PgsA for anchoring on the cell membrane of vegetative cell. The fusion genes were successfully integrated into chromosome of *B. subtilis* at *amyE* locus. Two promoters, P*rrnO* and P*gsiB*-P*secA*, were studied for expression of the fusion protein PgsA-PEDVS1. Protein expression study using Western blot

showed only cleaved product of PgsA-PEDVS1 protein in B. subtilis and this was in accordance with the PEDVS1 expression in *E. coli* BL21 (DE3), suggesting that PEDVS1 is an unstable protein in bacterial systems. To improve expression and stability, antigen need to be modified or re-designed.

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