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Research Article

CONSTRUCTION OF A BACULOVIRUS VECTOR CONTAINING A SUBUNIT OF PORCINE EPIDEMIC DIARRHEA VIRUS FOR PROTEIN DELIVERY

Nguyen Van Noi[1, 2], *Yao-Chi Chung[3] and Ta-Chih Cheng[1]
[1]*Department of Tropical Agriculture and International Cooperation, National Pingtung University of Science and Technology, Pingtung, Taiwan*
[2]*Institute of Life Science, Thai Nguyen University of Agriculture and Forestry, Thai Nguyen, Vietnam*
[3]*Graduate Institute of Animal Vaccine Technology, National Pingtung University of Science and Technology, Pingtung, Taiwan*
**Author for Correspondence*

ABSTRACT
The baculovirus can be used as a vector in gene delivery system. Viral envelope of baculovirus would display expressed protein and it could render as a potential vaccine delivery system. In this regard, the gene coding for a subunit of PEDV-S1 from E. coli strain was cloned in a baculovirus expression system. PEDV-S1 subunit has the ability to inhibit protein synthesis and this ability applied against Porcine epidemic diarrhea virus. In this study, expression of PEDV-S1 in baculovirus as a protein delivery system was assessed *in vitro*. PEDV-S1 gene was cloned in pFastBac HTb system expression vector. This vector enables the protein expression baculovirus. This construct was used to express the gene in baculovirus. The construct containing PEDV-S1 gene was made in baculovirus and expression was confirmed, then baculovirus expressing PEDV-S1 transfect Sf9 cells. The expression of pFastBacHTb_PEDV-S1 peptide (55kDa) was confirmed by SDS-PAGE and Western blotting in baculovirus expression system. The subunit challenge to Sf9 cells was applied as a delivery system by baculovirus. On the other hand, the inhibition of cell proliferation was also demonstrated by baculovirus containing PEDV-S1 subunit. PEDV-S1 peptide expression in baculovirus was shown in baculovirus expression system. Furthermore, it was shown that a subunit of PEDV-S1 delivered by baculovirus can inhibit cell proliferation in Sf9 cells and leading to cell death. Therefore, this prototype system could be a promising model for in vivo against PEDV and targeted protein delivery system.

***Keywords*:** *Baculovirus, Expression Protein, Porcine Epidemic Diarrhea Virus, Sf9 Cells*

INTRODUCTION
Porcine epidemic diarrhea (PED) is a devastating swine disease that is characterized by acute enteritis and lethal watery diarrhea followed by severe dehydration leading to death, with a high mortality rate in piglets (Debouck and Pensaert, 1980; Pijpers *et al.,* 1993). The disease was initially recognized in England in 1971, but the causative agent of this disease, PED virus (PEDV), was later identified in 1978 (Pensaert and De Bouck, 1978)**.** PED epidemics were first reported in Asia in 1982, and since then, PED has continued to threaten swine health, causing substantial economic losses in the Asian swine industry (Chen *et al.,* 2008; Li *et al.,* 2012b; Puranaveja *et al.,* 2009). In 2013, PED outbreaks suddenly occurred in the United States and have swept through the pork industry across the country, raising concerns about control measures for PED prevention (Mole, 2013; Stevenson *et al.,* 2013). In Taiwan, PEDV appeared in 2013 (Lin *et al.,* 2014); however, a retrospective study indicated that the virus had been present as early as 2013 (Lin *et al.,* 2013). Although periodic vaccination strategies have been implemented nationwide to control PED in Taiwanese swine herds, PEDV has continually emerged, causing tremendous harm to the productivity of Taiwanese pig farms.
PEDV, a member of the genus Alphacoronavirus within the family Coronaviridae of the order Nidovirales, is a large, enveloped virus possessing a single-stranded, positive-sense RNA genome of approximately 28 kb with a 50 cap and a 30 polyadenylated tail (Pensaert and De Bouck, 1978). The PEDV genome is composed of the 50 untranslated region (UTR), at least seven open reading frames

(ORF1a, ORF1b, and ORF2 through 6), and the 30 UTR (Kocherhans *et al.*, 2001). The two large ORFs 1a and 1b make up the 50 two-thirds of the genome and encode the non-structural replicase genes. The remaining ORFs in the 30 terminal region code for four major structural proteins: the 150–220-kDa glycosylated spike (S) protein, the 20–30-kDa membrane (M) protein, the 7-kDa envelope (E) protein, and the 58-kDa nucleocapsid (N) protein (Duarte *et al.*, 1994; Lee and Lee, 2014).

The S protein of PEDV is a type I membrane glycoprotein composed of 1,383 to 1,386 amino acids (aa), depending on the strain. It contains a putative signal peptide (aa 1–24), a large extracellular region, a single transmembrane domain (aa 1,334–1,356), and a short cytoplasmic tail. Although PEDV has an uncleaved S protein because it lacks a furin cleavage site, the S protein can be divided into S1 (aa 1–735) and S2 (736 - the last aa) domains based on homology with S proteins of other cor- onaviruses (Duarte and Laude, 1994; Jackwood *et al.*, 2001; Lee *et al.*, 2010b; Sturman and Holmes, 1984). Like other coronavirus S proteins, the PEDV S protein is known to play a pivotal role, interacting with the cellular receptor to mediate viral entry and inducing neutralizing antibodies in the natural host (Bosch *et al.*, 2003; Chang *et al.*, 2002).

More precisely, previous studies have shown that the S1domain includes the main neutralizing epitopes and the receptor-binding region (Lee *et al.*, 2011; Sun *et al.*, 2007). Furthermore, along with the full-length S gene, the S1 portion is known to be a suitable region for determining genetic relatedness among the different PEDV isolates and for developing differential diagnostic assays (Chen *et al.*, 2014; Lee *et al.*, 2010a). Considering these molecular and biological features of the S1 domain, it would be an appropriate target for developing effective vaccines against PEDV.

In this study, a subunit of PEDV was amplified and cloned in pFastBac HTb expression vector. Expressed recombinant S1 protein in baculovirus could be used as a cell proliferation inhibitory vehicle to infect cells and suppress the proliferation. This is the report of a subunit delivery into Sf9 cells by baculovirus. Further studies on in vivo models, could be more promising data for future clinical application.

MATERIALS AND METHODS

Bacterial Strains and Cell Culture

In this study, gene of PEDV-S1 was provided from Vaccine and Adjuvant Laboratory (Institute of Animal Vaccine Technology, National Pingtung University of Science and Technology, Pingtung, Taiwan). The DH5α, DH10Bac E. coli strains (ECOS 101 Yeastern Biotech, Taiwan) were used as hosts for the cloning.

Spodoptera Frugiperda (Sf9) cell lines were cultured at 27°C in TNMFH medium (PAN Biotech GmbH, Germany) with 10% heat-inactivated fetal bovine serum (PAN Biotech GmbH) and gentamicin (50 µg/ml) (PAN Biotech GmbH). Cell density and viability were assessed by Trypan blue staining. Cell viability was calculated on the basis of the percentage of living cells with respect to the total number of cells at various times post-infection. The Sf9 cells, which were cultured in suspension, were infected in spinner flasks (80 ml of culture media) at a cell density of 2x106 cells/ml. Cell viability at the time of infection was > 95% in monolayer and > 99% in suspension.

DNA Extraction

E. coli strains were grown on Luria-Bertani (LB) agar or in LB broth at 37^0C overnight. The DNA was extracted by Plasmid Miniprep Purification Kit (GeneMark, Taiwan). Pellet of bacteria culture by centrifugation for 1 min at top speed (12 - 14,000x g) in a microcentrifuge. Discard the supernatant and remove any excess media. Resuspend the cell pellet completely in 200 µl of Solution I by pipetting or vortexing. Add 200 µl of Solution II and mix by inverting the tube 5 times, the cell suspension should turn clear immediately. Add 200 µl of Solution III and mix by inverting the tube 5 times. Centrifuge the lysate at top speed in a microcentrifuge for 5 min. A compact white pellet will form along the side or at the bottom of the tube. Insert the spin column into a collection tube, carefully transfer all of the clear lysate to spin column, centrifuge at top speed for 1 min.

Discard the filtrate from the collection tube and add 700 µl of wash solution to the spin column and centrifuge at top speed for 1 min. Repeat this step once more. Discard the filtrate and centrifuge at top

speed for additional 3 - 5 min to remove residual trace of ethanol. Transfer the spin column into a new microcentrifuge tube and add 50 - 100 µl of elution solution or H_2O (pH 7.0 - 8.5) into the column and wait for 1 - 2 min. Centrifuge at top speed for 1 min to elute the DNA. Store the eluted plasmid DNA at - 20^0C. The DNA was used as a template for the amplification by the PCR (Maniatis *et al.*, 1982).

Polymerase Chain Reaction (PCR)

The PEDV-S1 gene was amplified by using the specific primers (Table 1). The forward primer with B*amH*I and the reverse primer with H*ind*III restriction enzymes were designed (Table 1).

Table 1: Forward and Reverse Primers used in this Study

Primer Sequences (Restriction Enzyme Sites in Underlined)	Restriction Enzyme	Primer
5'-GGGGGAATTCATGATTTCTTTTGTTACTCTGC-3'	B*amH* I	Forward
5'-TTTTGCGGCCGCCGCTGTAGAACATCCGTCT 3'	H*ind* III	Reverse

The PCR condition for the amplification was a follows: 5 min initial denaturation at 94ºC, followed by 30 cycles, each containing of 30 seconds at 94ºC, 30 seconds at 60ºC, and 1 min 30 seconds at 72ºC, and a final extension at 72ºC for 10 seconds. PCR was carried out in 10 µl volume containing 1 µl of DNA template, 5 µl of 2X Master mix, 0.5 µl of each primer, 3 µl of ddH_2O. The PCR products were loaded to agarose gel (1.2 %, w/v) for confirmation. The amplified fragment was cloned in pFastBac HTb vector (Invitrogen, USA). The subunit gene was sequenced and used for sub-cloning.

Construction of Expression Vector

In this study, the pFastBac HTb baculovirus expression vector (Invitrogen, USA) was used. Optimal protein expression in bacterial and insect cells can be done by a single plasmid. This vector uses tightly controlled T7*lac* promoter for expression in baculovirus-infected insect cells, the vector contain the *lef2*/603 and ORF1629 sites for recombinant into the baculovirus genome and use the p10 baculovirus promoter (Hitchman *et al.*, 2009) as mentioned in manual's protocol. The constructed vector was digested by *BamI* and *HindIII* restriction enzymes, sub-cloned in pFastBac HTb vector (Figure 1) and used for future study.

Production of Recombinant Baculovirus

After transformation of E. coli DH10Bac cells, Spodoptera frugiperda cells were cultured at 27^0C in TNMFH medium, supplemented with gentamicin (50 µgmL^{-1}). For transfection, 9 x 10^5 cells were plated in 6 well tissue culture dishes and incubated for 1 h in 2 ml Sf900-II SFM without antibiotics to allow adhesion of the cells to surface. Recombinant bacmid DNA had been preincubated for 45 min at room temperature with Cellfectin II (6 µl). Cells were incubated with the liposome-DNA complex for 5 h at 27^0C. The transfection medium was removed and 2 ml of Sf900-II SFM medium, containing antibiotics was added. The DNA was transfected into Sf9 cells. Transfected cells were incubated at 27^0C for 144 h allowing baculovirus production.

The recombinant virus was amplified twice to obtain virus stocks of the highest titer and harvested. Classical plaque assay was applied for virus titration (Summers and Smith, 1987).

Protein expression in Baculovirus

Sf9 cells in 6 well plates (TNMFH medium, 10 % v/v heat-inactivated FBS and gentamicin (w/v, 50 µgmL^{-1}) were infected by recombinant virus with MOI of 0.1, 0.25, 0.5, 0.75, 1 and 10 plaqueforming units per cell. Cells were harvested 1, 2, 3, 4, 5, 6 and 7 day post transfection and centrifuged for 5 min at 100 g. The cells were washed three times with phosphate-buffered saline (PBS) and then resuspended on ice in 300 µl of sonication buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 % (w/v) glycerol, IPEGAL CA-630) for 9 x 10^5 cells mL^{-1}. After sonication three times at 20 s pulses with a Bandelin model sonicator at 45 % power, samples were centrifuged at 4^0C for 10 min at 12,000g. PEDV-S1 activities were measured to determine optimum MOI with the maximum PEDV-S1 expression.

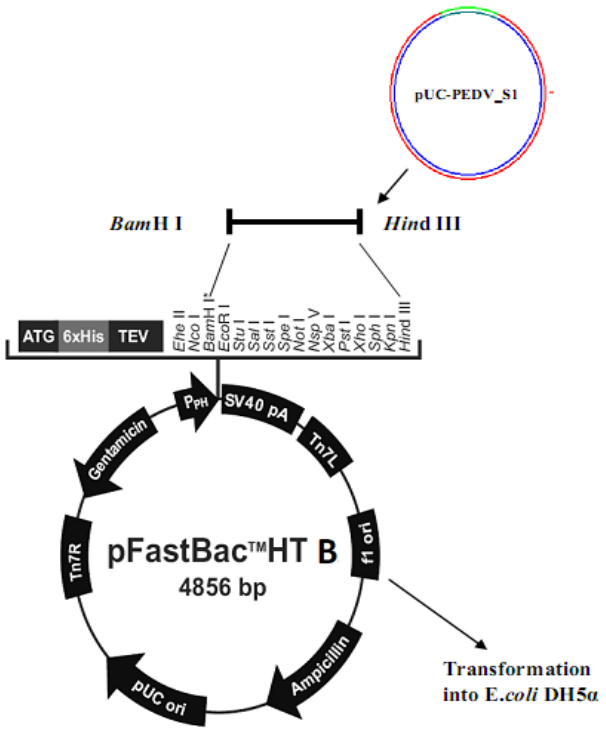


Figure 1: Restriction Map of the pFastBac HTb and a Subunit of PEDV-S1 Construct

SDS-PAGE and Western Blot

The bacterial and baculovirus harvested cell pellet suspended in lysis buffer and was assessed in 10% SDS-PAGE. After, electrophoresis they electro transferred to a PVDF nylon membrane (Roche). The expression was assessed in SDS-PAGE and confirmed by western blotting. Antibody against a subunit of PEDV-S1 that has already been raised was used for detection of protein expression. Detection was done by secondary antibody (Anti-rabbit horseradish peroxidase). For the horseradish peroxidase (HRP) enzyme, 3, 3>-Diaminobenzidine (DAB) was used as enzyme substrate. In each step, membrane was washed by PBST (0.1% Tween 20 and Phosphate-buffered saline) (Maniatis *et al.,* 1982).

Infection by Viruses

The ability of baculovirus to infect insect cells is depends on the titer of virus. Virus infectious titer (IT) could be measured by the plaque assay. For plaque assay, $1x10^6$ Sf9 cells were plated and added to each 6-well plates for 1h at room temperature. Cells were infected by (500µl) undiluted recombinant virus overnight and then the virus-containing medium was removed and replaced with fresh medium containing neutral red (0.5%, sterilized by filtration). Then the cells were incubated 3h at 37°C. After 1h incubation, 2ml of 1% agarose (final concentration) was added to medium containing serum as described in manual's instruction (Hitchman *et al.,* 2009). Transfection was monitored by staining of plaques in the monolayer after 3-4 days. Plaque identification depends on evaluation of several dilutions of virus with staining technique. In this experiment, Neutral Red (Merck) was used to stain live cells red, leaving clear plaques visible in the monolayer.

The total number of plaques can be calculated by multiplying the number of plaques observed on each plate by the dilution factor which is the inverse of the dilution used. Typically, plaque assay is expressed as plaque forming units per milliliter (pfu/ml). The corresponding virus dosage is hence expressed as multiplicity of infection (MOI) by considering the amount of virus and the number of used insect cells. Infected viruses with defined MOI were applied to Sf9 cell lines to measure the toxicity of construct. The cytotoxicity was measured by neutral red assay (Valdivieso-Garcia *et al.,* 1993).

RESULTS AND DISCUSSION

The plasmid construct was successfully transformed into *E.coli* DH5α cell and then DH10Bac and the obtained clones were assessed by PCR and digestion (Figure 2), which resulted in the generation of the bacmid DNA. The pFastBacHTb-PEDV-S1 expression vector transfected Sf9 cells to express PEDV-S1 in baculovirus. The pFastBacHTb-PEDV-S1 construct is able to express transgene in insect and vertebrate cells.

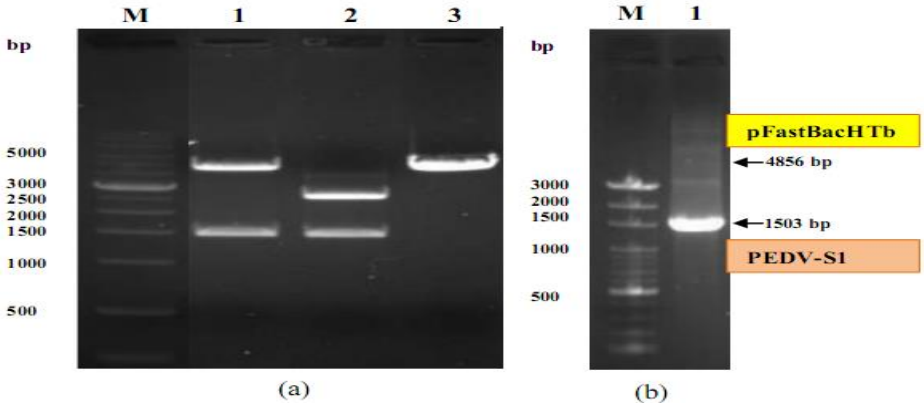
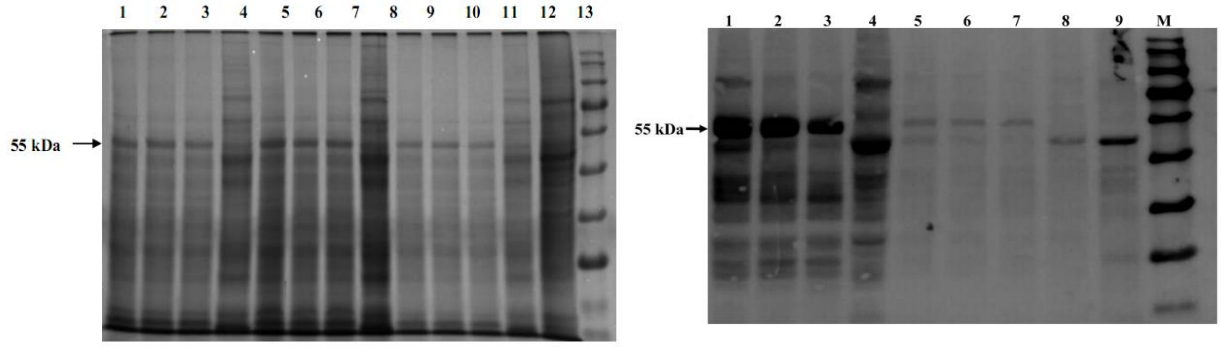


Figure 2: Purified Plasmid from Recombinant Clones was Digested by B*amH*I and H*ind*III Restriction Enzyme and Assessed by PCR in the Agarose Gel; (M: DNA Marker; lane 1: Correct-Size Digestion Products, Linearized pFastBacHTb-PEDV-S1 Gene; pUC-PEDV-S1 Gene; lane 3: pFast-Bac-HTb Vector).

Detection of Expression by SDS-PAGE and Western Blotting



(a) **(b)**

Figure 3: SDS-PAGE (a) and Western Blotting (b) of Expression in *E.coli* and Baculovirus
((a): Samples: Lane 1->3, lane 5->7; Lane 9->10; Lane 4, 8, 11: Cell only (Negative Control); Lane 12: E2 Protein (Positive Control); M: Protein Marker);
(b): Samples: Lane 1->3, Lane 5->7; Lane 4, 8: Cell only (Negative Control); Lane 9: E2 Protein (Positive Control); M: Protein Marker)

The recombinant clones with PEDV-S1 subunit was selected and induced by addition of IPTG. The expressed protein was analyzed on SDS-PAGE electrophoresis and detected by Western blotting. Western blot analysis was done by antibody against a subunit of PEDV-S1 that was raised previously (O'Reilly *et al.*, 1994). The induced clones in baculovirus was assessed and the subunit peptide as a 55KD protein was

observed (Figure 3). Expressions in baculovirus was detected with 1/20, 1/100, 1/500, 1/2500 dilutions as a folding dilution of the initial transfection mixture respectively, in the Sf9 cells. In Baculovirus expression one band was detected.

Infectious Titers of Baculovirus

Recombinant plaques were visualized 5-7 days after adding transfection mixture directly to a Sf9 monolayer insect cell (Figure 4). In figure 4 formation of plaques was shown grandually on monolayer of Sf9 cells, one day after adding virus to cells. Plaque formation was started, three, five days after adding virus dilution to Sf9 cells and it continues seven days after infection. The formed plaques were calculated to determine the infectious titer.

Total number of plaques was calculated by transfection and pfu/ml ($10 \times 10^3 - 2 \times 10^5$) was measured. The virus dosage 0.1 as corresponding to multiplicity of infection (MOI) was defined. Based on defined MOI, viral infection was calculated and cutotoxicity was assessed by adding baculovirus to Sf9 cells. The percent of cytotoxicity was calculated as follows: 1- mean absorbance of sample/ mean absorbance of negative control x 100. It was shown that the percentage of cytotoxicity was 30% for baculovirus infection. However, as a positive control, purified PEDV-S1 subunit showed 70% cytotoxicity on Sf9 cells. In this study, Mock treated cells were transfected with pFastBac HTb vector as a negative control.

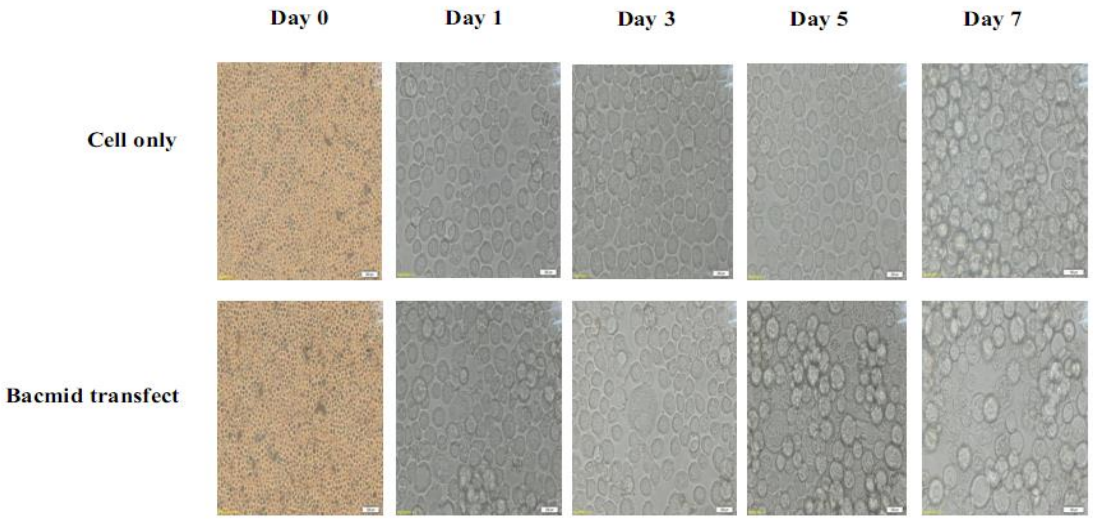


Figure 4: Plaque Assays; (a) Sf9 Cells Negative Control (Mock); (b) One Day after Virus Dilution Added to Sf9 Cells; (c) Three Days after Virus Dilution Added to Sf9 Cells; (d) Five Days after Virus Dilution Added to Sf9 Cells; (e) Seven Days after Virus Dilution Added to Sf9 Cells. Plaques are shown by the Arrows in the figure

Recently, baculovirus expression system and Sf9 cell line have widely used for recombinant protein production. The PEDV-S1 gene was successfully expressed in the baculovirus-insect cell system in a related study (Ko *et al.*, 2011). Examined parameters, infection time (0, 1, 2, 3, 4, 5, 6 and 7 day) in this study. The recombinant protein production has been widely employed in the insect host cells. Baculovirus as an insect virus naturally infects and replicates in the host insect cells (Hu, 2005). Baculoviruses has been used in biotechnology applications beyond the production of proteins in insect cells (DeMaria *et al.*, 2000).

PEDV-S1 has been applied as fusion products to target the protein to specific cells or direct injection of PEDV into tumors to kill cells. PEDV-S1 application as a fusion protein is complicated because any cell, tumor, or normal that comes into contact could be killed (Gomez-Sebastian *et al.*, 2014). Expression of recombinant in insect cells could apply as a new vehicle to effectively deliver PEDV-S1 subunit gene into cells.

One of the most advantages of baculoviruses is its production in insect cells. There are many advantages of Baculovirus vectors usage, for gene therapy. Collectively, baculovirus vectors usage are large cloning capacity, ease of production, lack of toxicity and replication, and lack of pre-existing immunity (Sung *et al.*, 2014).

In this regard, a recombinant baculovirus vector was designed carrying gene encoding a subunit of PEDV-S1. The expression of PEDV-S1 subunit was assessed in insect cells. The expression in baculovirus system was confirmed. One corresponding band was observed for expressed protein in baculovirus.

Presumably, baculovirus expression is along with co- and post translational of the expressed proteins that result to the different size of the protein (Li *et al.*, 2012a; Richardus and Grp, 2013). However, baculovirus carrying the construct, infected Sf9 cells and the cytotoxicity was investigated. It was shown that S1 subunit of PEDV was successfully delivered to the cells. Protein delivery by baculovirus is the safest way of virus delivery system (Huy *et al.*, 2011).

Moreover, baculoviruses should, more be used for targeted against PEDV (Cao *et al.*, 2015). In the future, advantages and disadvantages of baculoviruses usage will be more defined as vehicles for delivery of gene/protein to Sf9 cells.

This study is the prototype pilot study to construct a baculovirus vehicle containing PEDV-S1 for the first time and using baculovirus infection capability to infect Sf9 cells as a tool for gene/protein delivery. PEDV-S1 cytotoxicity and its use in mice, pigs as in vivo model could provide additional data for any future application. Further studies on in vivo models, would provide more promising data for future clinical application.

Conclusion

The PEDV-S1 peptide expression in baculovirus was shown in baculovirus expression system. Furthermore, it was shown that a subunit of PEDV-S1 delivered by baculovirus can inhibit cell proliferation in Sf9 cells and leading to cell death. Therefore, this prototype system could be a promising model for in vivo against PEDV and targeted protein delivery system.

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