Asian Journal of Biological and Life Sciences | Original Research

Development of a recombinant S1 domain of the porcine epidemic diarrhea virus spike protein based ELISA for virus detection

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Submitted : 14.05.2016 Accepted : 13.08.2016 Published : 30.08.2016

Abstract

A recombinant S1 domain of the porcine epidemic diarrhea virus spike protein (S1), protein-based ELISA was developed to detect porcine epidemic diarrhea virus (PEDV). The S1 gene of PEDV was expressed in *Escherichia coli*. The purified recombinant S1 protein was used to immunize rabbits to generate a polyclonal antibody. Immunofluorescence analysis indicated that the anti-PEDV-S1 antibody reacted with PEDV-infected cells. The antibody was utilized to develop an indirect ELISA to detect PEDV. Other viruses, porcine transmissible gastroenteritis coronavirus, avian infectious bronchitis coronavirus, porcine reproductive and respiratory syndrome virus, classic swine fever virus and porcine pseudorabies virus, were unreactive.

Key words : Coronavirus, PEDV-S1 protein, Porcine epidemic diarrhea virus

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is an acute and highly contagious enteric infectious disease characterized by vomiting, diarrhea, and dehydration in pigs of all ages, but especially in newborn piglets [1,2]. PEDV was first reported in England in 1971[3] and then detected in Japan in 1982 and subsequently confirmed in other southeastern Asian countries[4]. In the USA, PEDV was first reported in 2013 and has since rapidly spread throughout the country [5,6]. In China, the incidence of PEDV outbreaks has rapidly increased since 2010, especially among newborn piglets aged from a few hours to one week, often resulting in death due to watery diarrhea and dehydration [7,8]. Although the use of inactivated and attenuated vaccines may have helped to reduce the prevalence of disease, PEDV has continually emerged, causing tremendous losses to the swine industry in China [9].

PEDV, a member of the genus Alphacoronavirus in the family Coronaviridae, has a single-stranded positive-sense RNA genome of approximately 28 kb encoding four structural proteins: the spike (S), nucleocapsid (N), envelope (E), and membrane (M) proteins [10,11]. The S protein is a glycoprotein consisting of 13831387 amino acids (aa) on the viral surface, which is composed of four regions, a signal peptide (aa 124), an extracellular region, a transmembrane domain (aa 1,3341,356), and a short cytoplasmic tail (aa 1,3571,383) [12]. Unlike other coronaviruses (i.e., murine coronavirus and bovine coronavirus), the S protein of PEDV cannot be cleaved into S1 and S2 domains after PEDV maturation. Thus, the S1 domain (residues 1789) and S2 domain (aa 7901383) of PEDV are artificially defined based on homology to other coronavirus S proteins [13,14]. The S protein is responsible for binding and fusion of the virus to the host cells. It therefore not only determines cellular tropism but also plays a vital role in inducing production of neutralizing antibodies in the host [15,16], and the recombinant S1 protein was previously shown to have protective activity in piglets [17]. Therefore, the S1 protein may be used as a candidate diagnostic reagent for determining PEDV infection.

In this study, we have expressed the S1 gene of PEDV and generated an anti-S1 polyclonal antibody via animal inoculation. Analysis on the activities of the S1 protein and the antibody indicated that both proteins were biologically active. The antibody was able to discriminate PEDV from other viruses selected.

MATERIAL AND METHODS

Construction of expression plasmid, pRSET-A_PEDV-S1

A recombinant plasmid pRSET-A_PEDV-S1 containing the full-length S1 gene of PEDV was used as a template to amplify a signal peptide sequence-deleted S1 gene using a sense primer: T7PF'-TAATACGACTCACTATAGGG and a reverse primer: T7TR'-CTAGTTATTGCTCAGCGGTGG. Both primers contained EcoRI and XhoI sites (underline parts), respectively. The PCR parameters included 94^{0}C for 5 min, 30 cycles of 94^{0}C for 30 sec, 60^{0}C for 30 sec, 72^{0}C for 1 min 30 sec and a final extension of 72^{0}C for 10 sec. The PCR product was cloned into the same sites of a prokaryotic expression vector, pRSET-A (Invitrogen, USA), resulting a recombinant plasmid pRSET-A_PEDV-S1. The DNA plasmid was purified using a Plasmid miniprep purification kit (GeneMark, Taiwan) prior to DNA sequencing.

Expression and purification of the protein of interest

The pRSET-A_PEDV-S1 was transformed into host cells [E. coli BL21(DE3) pLysS] and the protein expression was analyzed according to a recent reference with modifications [18]. Briefly, the bacteria harboring the pRSET-A_PEDV-S1 were cultured in Luria-Bertani (LB) medium at 37^{0}C with shaking until the OD_{600} reached 0.5. Then, IPTG was added to 0.5 or 1 mM to induce protein expression at 37 or 30^{0}C for 6 h. Empty vector-transformed bacteria were used as control. Expression amount of

the S1 protein in the total bacterial protein was analyzed using software. The bacterial suspension (20 ml) was centrifuged; the cells were re-suspended in TE buffer (50 mM Tris/ HCl and 1 mM EDTA, pH 8.0), mixed with lysozyme (100 µg/l) at room temperature for 30 min, then was sonicated on ice for 30 min and finally centrifuged at 10,000 xg for 20 min. The supernatant and pellets were mixed with SDS-loading buffer, subjected to 10% (v/v) SDS-PAGE and the gels were stained with Coomassie brilliant blue. The gel purification and re-naturation of fusion protein by dialysis were performed according to Liu et al. (2009). The protein of interest was designated as PEDV-S1.

Preparation and titration of anti-S1 protein polyclonal antibody

To generate a specific polyclonal antibody against the PEDV-S1 protein, two New Zealand rabbits were immunized with the purified PEDV-S1 protein (2 mg/ml) emulsified with Freund's complete adjuvant via subcutaneous injection (1 ml/each rabbit). Two weeks later, the rabbits were injected with the same antigen (0.5 ml) mixed with Freund's incomplete adjuvant. Subsequent injections were made every week for 10 weeks. Blood of the immunized rabbits was collected from neck artery and the anti-M antibody was isolated. The antibody titer was determined using ELISA. Briefly, ELISA plates were coated with the PEDV-S1 protein (1 lg/well) at 4^0C and held overnight in 50 mM Na2CO3/NaHCO3 buffer, pH 8.6. The next day, the wells were blocked with 5% (w/v) non-fat dry milk in PBS/0.05% Tween 20 (PBST) at 37^0C for 2 h, after washing three times with PBST. Then the wells were incubated with serially diluted polyclonal antibody at 37^0C for 1 h. Serum from a non-immunized rabbit was used as control. The wells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000 diluted in PBS) at 37^0C for 1 h followed by addition of o-phenylenediamine dihydrochloride [19] for 10 min. The reaction was stopped using 2MH2SO4 and the A450 value was read using an ELISA plate reader.

Western blot

E. coli expressing either the PEDV-S1 protein or empty vector was induced by IPTG and the lysates were subjected to 10% (v/v) SDS-PAGE. The proteins were electronically transferred to a nitrocellulose membrane which was blocked overnight at 4^0C using 5% (w/v) non-fat dry milk in PBST buffer and then incubated with anti-S1 polyclonal antibody (1:400 dilution in PBST) at room temperature for 2 h. After the membrane was washed three times with PBST, they were incubated with HRP-conjugated goat anti-rabbit IgG (1:2,000 dilution in PBST) for another 1 h. The protein bands were visualized using OPD.

Immunofluorescence

African green monkey kidney cell line, Vero cells (ATCC CCL-81) were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 5% (v/v) fetal bovine serum at 37^0C. The next day, the cells were infected with PEDV isolate TW1/A (KJ434294) at 37^0C for 48-72 h. Immunofluorescence assays were performed [20,21] with modifications. Briefly, the cells were fixed 4% (w/v) formalin in PBS for 20 min and then were quenched with 0.1 M glycine in PBS. Then they were incubated with the anti-S1 antibody at 1:100 in 1% BSA at 37^0C for 1 h. The cells were incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Sigma), 1:200 diluted in 1% BSA for another 1 h in the dark. After washing three times with PBS, the fluorescence was observed under a fluorescence microscope.

ELISA

ELISA plates were coated with a panel of viruses. The coating concentration was 2 µg per well at 4^0C overnight. The next day, the wells were blocked with 5% (w/v) non-fat dry milk-PBST at 37^0C for 2 h. The anti-PEDV-S1 protein antibody (1:100 diluted in PBST) was added into the wells at 37^0C for 1 h. After rinsing three times with PBST, the wells were incubated with HRP-conjugated goat anti-rabbit IgG (1:1,000 diluted in PBST) at 37^0C for 1 h.

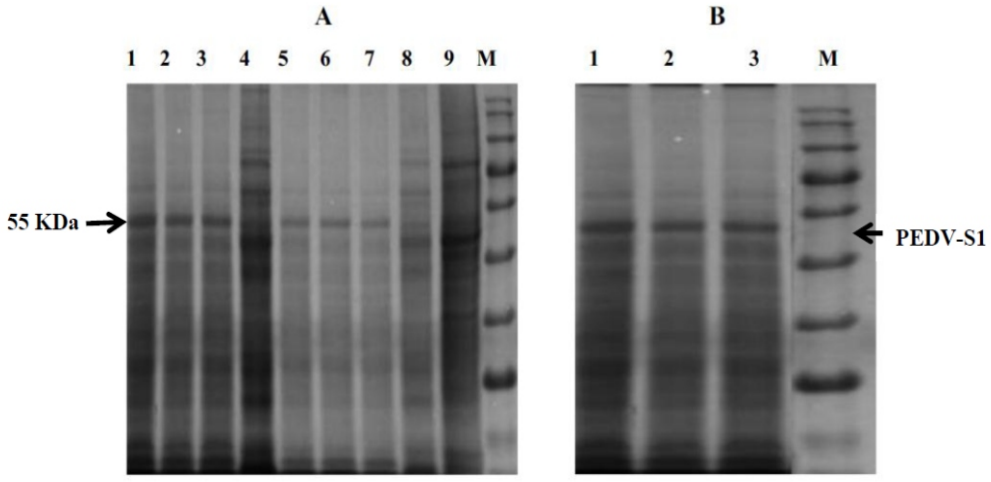

Fig 1: Expression of PEDV-S1 protein in E. coli. The bacteria containing either PEDV-S1 gene or empty vector were induced by 0.5 mM IPTG at 370C. The S1 protein expression was determined every hour using SDS-PAGE (A). Lane M: Protein molecular weight marker, Lane 4, 8, 9: Uninduced bacteria, Lanes 1, 2, 3: Lysates of empty vector transformed bacteria at 16 h post-IPTG induction, Lanes 5, 6, 7: Expressed S1 protein at 15 h post-IPTG induction. The bacteria were lysed and centrifuged. The supernatant and pellets were subjected to SDS-PAGE (B). Lane M: Protein molecular weight marker, Lane 2, 3: Expressed S1 protein at 6 h post-induction, Lane 1: Supernatant and pellets of lysed bacteria containing the S1 gene at 6 h post-induction, respectively. Arrows indicate the position of the S1 protein with a molecular weight of approx. 55 kDa

OPD was added and incubated for 15 min after washing with PBST. The A_{450} was read using an ELISA plate reader.

RESULTS

Construction and expression of the recombinant plasmid bearing PEDV-S1 gene

The signal peptide sequence-deleted PEDV-S1 gene was amplified using PCR and then cloned into the prokaryotic expression vector. The authenticity of the insert was confirmed by DNA sequencing. The cloned gene was identical to the reported S1 gene sequence of PEDV strain TW1/A (GenBank accession number: KJ434294). SDS-PAGE analysis indicated that expression of PEDV-S1 protein was detectable at 1 h post-IPTG induction (Fig 1A). The protein was expressed as inclusion body in recombinant E. coli and its molecular weight was approx. 55 kDa as expected (Fig 1B). The optimal induction condition for PEDV-S1 protein expression was 0.5 mM IPTG at 37^0C. The expressed S1 protein was then purified (Fig 2).

Production and biological analysis of the antiPEDV-S1 antibody

The purified S1 protein was used to immune rabbits for generating a polyclonal antibody. The antibody titer was $1:10^{12}$. To analyze the recognizing ability of the anti-PEDV-S1 antibody to the S1 protein, the antibody was used as primary antibody in a conventional Western blot. The polyclonal antibody reacted with the S1 protein (Fig 3). The biological activity of the antibody was further analyzed using an immunofluorescence assay. Green fluorescence was detected in the PEDV-infected cells (Fig 4).

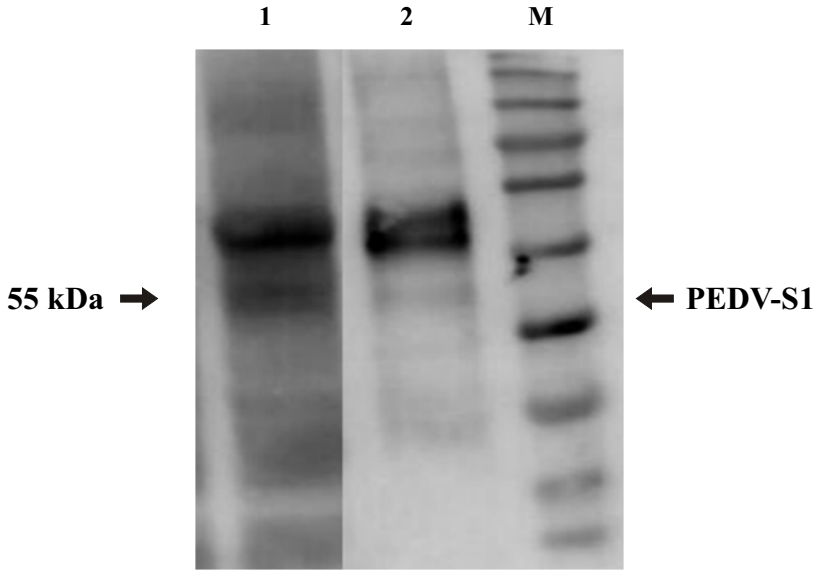

Fig 2: Purification of PEDV-S1 protein. The expression of PEDV-S1 protein in E. coli was induced by 0.5 mM IPTG at 370C for 4 h and the gel was stained with 0.25 M cold potassium chloride for 30 min followed by gel purification. The unpurified bacterial lysate and purified S1 protein were analyzed by SDS-PAGE. Lane M Protein molecular weight marker, Lane 1 Unpurified bacterial lysate, Lane 2 Purified S1 protein.

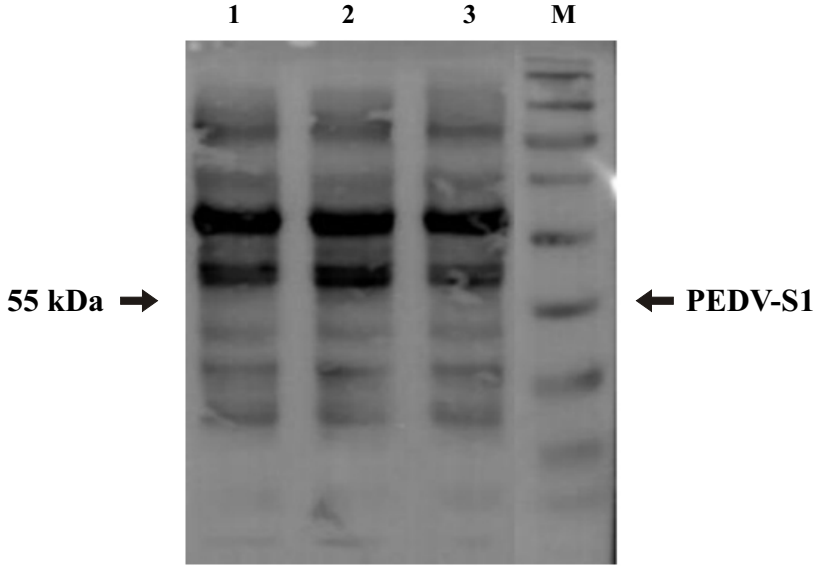

Fig 3: Western blot analysis of PEDV-S1 protein. After the proteins from empty vector or PEDV-S1-transformed bacteria were transferred on a nitrocellulose membrane, a conventional immunoblotting was performed using anti-S1 antibody generated in this study as primary antibody. Their Western blot results are shown in lanes 1, 2, 3. Lane M Protein molecular weight marker.

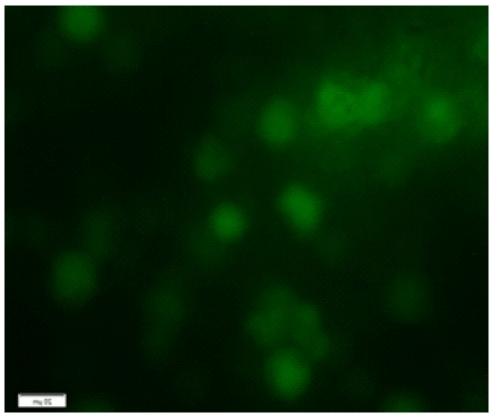

Fig 4: Detection of PEDV-infected cells using immuno fluorescence assay. PEDV-infected Vero cells were subjected to indirect immunofluorescence assays and the detection of the PEDV by the anti-S1 protein antibody is shown. Scale bar is 20 µm.

ELISA discriminating PEDV from other viruses

To investigate the utility of the antibody further, various viruses were used as coating antigens in indirect ELISA. Our results indicated that the anti-S1 antibody reacted with PEDV exclusively and no cross-reaction between the antibody and other viruses was detected [22].

system is a time- and cost-efficient system for high-level protein production. Selection of a suitable prokaryotic system is important for foreign gene expression. We have already expressed several heterologous proteins, including glycoproteins, in prokaryotic systems and the bacterially-expressed proteins display excellent biological activities [18,26].

High-level prokaryotic expression of envelope exterior of PEDV-S1 protein has been reported [27]. In this study, we expressed full-length PEDV-S1 gene using a similar expression system. Availability of the mature M protein may allow us to identify more antigenic epitopes in this protein and make more functional analysis in the future. The S1 protein of PEDV has been identified by use of rabbit anti-peptide sera and transient expression of the S1 gene in Vero cells and by expression in the baculovirus system. The native S1 protein of PEDV is incorporated into virions, is N-glycosylated and with a molecular size of 55 kDa. In contrast, the S1 protein synthesized by recombinant baculoviruses migrates with a PEDV-rS1-Ig of 170 kDa that is identical with the deglycosylated product of PEDV. M protein specified by the recombinant baculovirus is poorly, if at all, glycosylated [28]. In this study, the bacterially-expressed S1 protein is excluded from glycosylation and also migrates with a PEDV-rS1-Ig of 170 kDa (excluding the His-tag in the N-terminus). However, amount of the protein is enough for functional analysis and related experiments. The S1 protein was inoculated into rabbits to generate antibody. At different immunization intervals, we found that the titer of the anti-S1 antibody increased slowly, therefore,

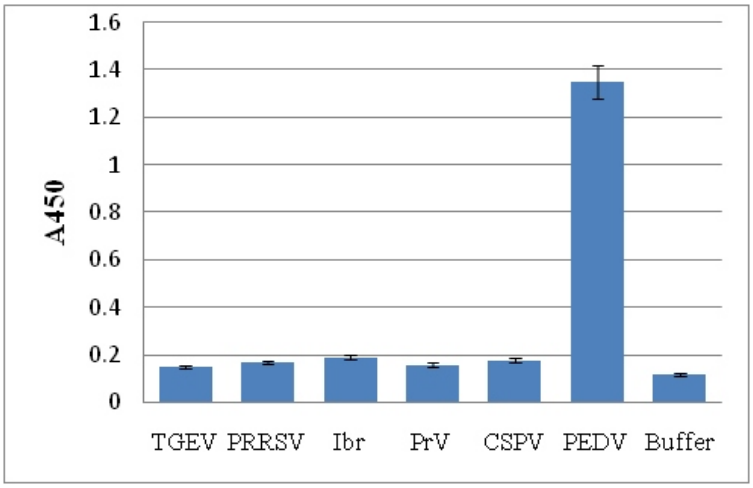

Fig 5: Discrimination of PEDV from other viruses in ELISA. The anti-PEDV-S1 antibody produced in this study was used as primary antibody in ELISA to detect a panel of viruses. Virus lysis solution was used as control buffer. A450 is shown in y axis. PEDV, transmissible gastroenteritis coronavirus (TGEV) infectious bronchitis coronavirus (IBV), porcine reproductive and respiratory syndrome virus (PRRSV), classic swine fever virus (CSFV), porcine pseudorabies virus (PrV) and buffer are indicated in x axis.

DISCUSSION

The first Chinese PEDV was isolated in 2013. Since then more PEDV infections have been reported in different areas and PED has been a serious threat to the pig industry in Taiwan. Effective diagnostic methods are required for surveillance of PED. The first purpose of the current study was to express PEDV-S1 gene. The expression of coronavirus S1 protein is difficult due to its sprout function. The viral S1 protein may cause damage to cell wall resulting in the growth inhibition [23]. PEDV-S1 protein has been expressed in eukaryotic cells and a baculovirus system [24,25]. However, low expression and high costs may affect the application of the expressed protein. In contrast, the prokaryotic

we had to prolong the immunization period (up to 3 months) to achieve a higher antibody titer. In our study, the sera collected from two immunized rabbits gave the similar antibody titer. Western blot and immunofluorescence assays showed that the antibody recognized the S1 protein and the virus-infected cells, indicating that the polyclonal antibody can be used for detection of PEDV.

Enteric infection caused by PEDV should be differentiated from other enteric viral diseases such as TGEV, a counterpart of the same group in the family Coronaviridae. They caused similar clinical signs and histological intestinal lesions, such as severe villus atrophy in the proximal and distal jejunum as well as in the

ileum [29]. Herein porcine coronaviruses (TGEV and PEDV), avian coronavirus (IBV), porcine arterivirus (PRRSV), porcine pestivirus (CSFV) and porcine herpesvirus (PrV) were used as coating antigens in an indirect ELISA. The viruses have been confirmed by ELISA or PCR.

CONCLUSION

The results showed that the anti-S1 polyclonal antibody was specific to PEDV. These selected viruses are common pathogens in Taiwan and some of which may cause mixed infection with PEDV. The established ELISA described in this paper is valuable for differentiating infection between PEDV and other related viruses.

ACKNOWLEDGEMENT

The authors deeply thank Prof. Dr. Chun-Yen Chu for her critical comments and the help in the preparation of this research paper.

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