



Expression of recombinant classical swine fever virus *E2* glycoprotein in stable High Five cells

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Classical swine fever (CSF), a notified disease by the World Organization of Animal Health (OIE), also known as hog cholera or pig plague, is an extremely contagious, often fatal disease of swine that spreads in both epizootic and enzootic forms. The disease is caused by classical swine fever virus (CSFV) that belongs to the genus Pestivirus in the family Flaviviridae (Moennig 2000). CSF causes huge economic losses to the pig industry worldwide due to restrictions on the trade of pig and pig products infected by CSF (Postel *et al.* 2019; Ito *et al.* 2019). CSF is endemic in India. The majority of states in India have reported CSF outbreaks (Patil *et al.* 2021). The other members of the genus Pestivirus include BVDV and BDV (Righi *et al.* 2021). The genome of the virus is a positive sense, single-stranded RNA of approximately 12,300 nucleotides and flanked by 5' and 3' non-translating regions (NTRs) (Elbers *et al.* 1996). The proteins are arranged in the order Npro/C/Erns/E1/E2/P7/NS2-3/NS4A/NS4B/NS5A/NS5B. The *Erns*, *E1*, and *E2* are major glycoproteins located in the outer membrane of the virion. These glycoproteins are involved in virus attachment and penetration into the host cells. Among them, the *E2* glycoprotein is highly immunogenic and induces neutralizing antibodies (Maurer *et al.* 2005, Van Rijn *et al.* 1996). The *E2* glycoprotein has been targeted for the development of immuno-diagnostics as well as immuno-prophylactics against CSFV. Keeping in view the above facts the present study was designed to clone and express the codon-optimized CSFV *E2* gene in the High Five insect cell line and characterize the recombinant *E2* protein for its diagnostic potential.

Cloning of CSFV *E2* gene in insect cell expression vector: The antigenic index of the *E2* protein was analyzed by using the Protean program of DNASTAR Lasergene software, USA. 1008 bp *E2* coding sequences (CDS)

were selected for cloning and expression following codon optimization and synthesized commercially from BIO BASIC. The laboratory-modified insect cell expression vector (pI-SP) carrying a secretory signal peptide at the 5' end and a 6X His tagged sequence towards the 3' end of the multiple cloning site was used to clone the PCR amplified *E2* gene with the help of *E2*-pI-F: 5'- GTGGAATTCGCCATGGGTAGGTTGGC TTGTAAGGAGGACT -3' and *E2*-pI-R: 5'-TGAGCGG CCGCCCACATCCAGATCAAACCAGTAT-3' sets of primers (Invitrogen, Thermo fisher scientific, USA). The PCR product of the amplified CSFV *E2* gene was purified by using a commercially available extraction kit (Wizard SV Gel and PCR Clean up system, Promega).

RE digestion of gel-purified PCR product of CSFV *E2* gene and pI-SP vector for cloning: The purified PCR product of CSFV *E2* gene (insert) and laboratory-modified pI- SP plasmid were subjected to *EcoR*I and *Not*I double RE digestion, separately. The RE-digested pI-SP vector and CSFV *E2* fragments were gel purified separately by using a commercial gel extraction kit (Wizard SV Gel and PCR Cleanup system, Promega).

Ligation and transformation: The RE digested and gel-purified, CSFV *E2* gene fragment was ligated to pI-SP vector in an insert vector molar ratio of 1:2 using T4 DNA ligase overnight at 22°C in a PCR thermocycler. Then the ligation mixture was used to transform into *E. coli* top ten competent cells by heat shock method at 42°C for 50 s in a pre-warmed water bath.

Screening of recombinant clones by colony-PCR: The clones (10-15 Nos) obtained after transformation, were picked and streaked on the fresh LB agar plates containing Zeocin (25 µg/ml) and incubated at 37°C for 16 h. Overnight streaked clones were used as templates for screening of recombinant clones with desired gene insert (CSFV *E2*) using vector backbone specific sequencing primers, i.e. OpIE2-F and OpIE2-R (Invitrogen, Thermo Fisher Scientific, USA).

Expression of CSFV *E2* gene in High Five insect cell Transfection of pI-SP-*E2* recombinant plasmid into

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High Five cells: The endotoxin-free plasmids carrying the E2 gene were extracted by using a commercial kit (MACHEREY- NAGEL). The High Five cells cultured in the Express Five serum-free medium were used for the expression of the secreted protein, i.e. CSFV E2 using a cationic lipid-based transfection method in the duplicate well of a 6-well cell culture plate. Cellfectin II reagent (Invitrogen) was used for the expression of recombinant plasmid with a molar ratio of 3:1, i.e. (9 µl cellfectin and 3 µg of plasmid) in a 6-well cell culture plate. The recombinant plasmids carrying the E2 gene with secretory signal peptide along with a positive control vector containing green fluorescent protein (EGFP) were used to transfect High Five cells.

Development of a stable High Five cell line constitutively secreting CSFV E2 protein: The transfected High Five cells were incubated with selection media (Growth media including zeocin antibiotic (150 µg/ml) after 72 h post-transfection and the selection medium was replaced every 3rd day in the transfected six-well plate. Later on, within 3-4 weeks, the selected cells were sub-cultured and transferred to a 25cc culture flask, and the same zeocin concentration was maintained to develop a stable cell line. After 6-7 weeks of transfection, the stable High Five cells were grown with a reduced concentration of zeocin @50 µg/ml just to maintain the selection pressure. Some fraction of the transformed and growing High Five stable cells was cryopreserved at Liquid nitrogen using a cell freezing medium.

Harvesting of E2 recombinant protein for expression analysis: Cell Culture supernatant was harvested from a 25cc flask at 72 h of culture of the stably transfected High Five Cells and collected into a 15 ml centrifuge tube. The collected supernatant was centrifuged at 5000 rpm, 4°C for 10 min. to remove cellular debris. The supernatant was then filtered through a 0.45 µm syringe filter. The sterilized supernatant was then dialyzed using 1 L sterile PBS (pH 7.4) at 4°C overnight using Snakeskin Dialysed tubes (12 kDa cut-off). The dialyzed protein was then concentrated using PEG 6000 over the dialyzed bags for 4-6 h. The concentrated CSFV E2 protein was collected from the dialysis tube and protein concentration was estimated using the Q-Bit system (Invitrogen Thermo Fisher Scientific, USA).

Adaptation of stably transfected High Five cells in suspension culture for production of E2 recombinant protein: 250 ml screw-capped conical flask was siliconized (2% dimethyldichlorosilane in chloroform) to prevent the cells from sticking to the glass and used for subculturing the transformed High Five cells. Express Five media (~25 ml) with 5% serum (Fetal bovine serum) and fresh Zeocin (50 µg/ml) were then added. The flask was incubated at 26°C at a constant stirring rate of 80 rpm. The cells started growing well after 24 h onward. After 48 h of culture, the cells were counted by hemocytometer and their viability and cell density were determined. Then gradually, over a period of 2 weeks, the percentage of serum was reduced

as the cells were adapted to suspension culture conditions. After 2 to 3 weeks, the cells were considered adapted to suspension culture when >90% viability of cells was observed. The cells were cryopreserved for future use.

Purifications of CSFV E2 recombinant protein by FPLC (AKTA prime chromatography system): The size exclusion chromatography was done by using AKTA prime system and HiPrep™ 16/60 Sephacryl® S-100 HR column was used to recover the expressed E2 protein using sodium phosphate buffer.

SDS-PAGE analysis of purified CSFV E2 recombinant protein: SDS-PAGE analysis was carried out in a vertical mini gel electrophoresis apparatus (Bio-Rad). The samples (CSFV E2 protein) used were purified and concentrated then analyzed in SDS-PAGE.

Western blot and Dot blot analysis of the recombinant envelope glycoprotein: Expressed recombinant CSFV E2 protein was characterized by western blot and dot blot analysis to confirm the specificity/reactivity with CSFV antisera as well as monoclonal antibody. CSFV antisera was provided by Department of Microbiology, COVSC, Khanpara, Guwahati, Assam, India. Monoclonal antibodies used were CSFV-Specific mABWH303, procured commercially from Creative Biolab.

Classical swine fever (CSF) is a highly contagious and economically significant viral disease of pigs. The E2 gene is considered essential for CSFV replication (Van Gennip *et al.* 2002). E2 has been implicated, along with Erns and E1 in viral adsorption to host cells and also CSFV virulence (Borca *et al.* 2019). Bacterial culture carrying codon optimized CSFV E2 gene in pUC57 vector (pUC57-E2) and insect cell expression vectors pI-SP were grown overnight at 37° C. Then the PCR product as well as the pI-SP expression vector were subjected to double RE digestion with *Eco RI* and *Not I*. RE could successfully release the codon-optimized CSFV E2 gene insert with a product size of 1010 bp and linearized the pI-SP expression vector with a product size of 2877 bp as evidenced in agarose gel electrophoresis (Supplementary Fig. 1). The set of primers amplified the vector backbone with the gene of interest from the expression vectors pI-SP-E2. PCR could amplify the CSFV E2 gene with a product size of 1267 bp from pI-SP-E2 recombinant plasmid as revealed by 1% agarose gel (Fig.1).

The Baculovirus Expression Vector System (BEVS) is considered one of the most successful and widely acceptable means for the production of recombinant proteins in extremely large quantities in insect cells. Proper post-translational modifications of the expressed proteins in insect cells, the usual host of baculovirus, get them soluble, correctly folded, and biologically active products (Amer 2011). Autographa californica (AcNPV) is the most widely used expression vector. The most widely used hosts for AcNPV were the established insect cell lines IPLB-Sf21- AE (Sf21) and SfH9, originally derived from *Spodoptera frugiperda* ovaries (Vaughn *et al.* 1977), and BTI-TN-5B1-4 (High-Five), an insect cell line derived

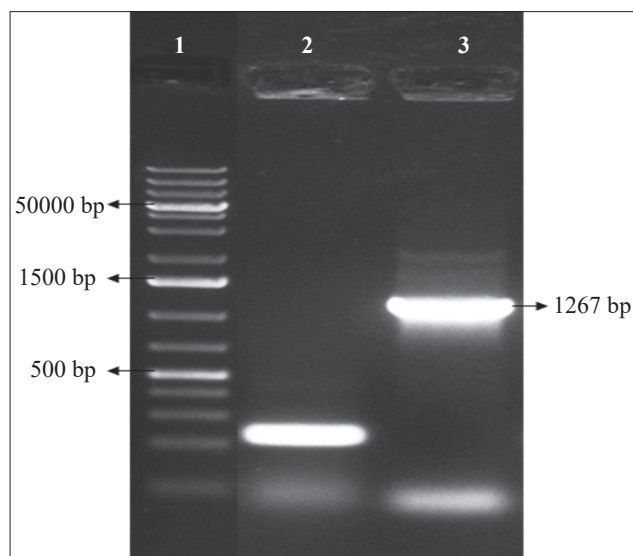


Fig. 1. Screening of recombinant pI-SP-E2 clones by colony PCR [Lane 1: Marker (1 kb plus DNA ladder); Lane 2: Negative clone; Lane 3: Positive clone].

from *Trichoplusiani* eggs (Wickham *et al.* 1992). Stably transfected cell lines are alternatives to transient protein production. It also has limitations of low protein yield and this is time-consuming process (Harrison and Jarvis 2007).

To overcome these limitations, the rapid production of recombinant proteins from mammalian cells gene expression is now a well-established technology. Gene expression technology is based on the transfection of cells with plasmid expression vectors (Backliwal *et al.* 2008, Daramola *et al.* 2014). GE has also been demonstrated with adherent sf9 cells using either cationic lipids or polymers for DNA delivery (Li *et al.* 2001, Loomis *et al.* 2005, Ogay *et al.* 2006). Shen *et al.* (2014) described a method in which yields reaching up to 100 µg/ml were achieved at the 300 ml scale for a secreted protein described for large-scale Transient gene expression (TGE) with suspension-adapted Sf9 cells using polyethylenimine (PEI) for plasmid DNA delivery. For stable as well as transient protein production High Five cells have several advantages over Sf9 cells. High Five cells as the host consistently demonstrated higher yields as compared with Sf9 cells in BEVS for the transient production of several secreted proteins (Krammer *et al.* 2010, Wilde *et al.* 2014). High Five cells have higher transfection efficiency of up to 90% (defined by the percentage of EGFP-positive cells) and a TNFR- Fc yield of 150 µg/ml at volumetric scales up to 300 ml was achieved (Shen *et al.* 2015).

In this study, for the production of stable secretory recombinant protein, a CSFV *E2* gene expression was attempted in an insect (High Five) cell expression system using a Cellfectin II cationic reagent. Endotoxin-free plasmids were prepared from the pI-SP-E2 clones by using a commercial kit. The purified pI-SP-E2 plasmid was quantified using nanodrop (Thermo) and then used to transfect High Five cells at around 70-80% confluence. An enhanced green fluorescent protein (EGFP) expressing

plasmid (pI-EGFP) was also transfected as a reporter to measure the efficacy of transfection by observing the green fluorescence under the microscope.

Following 72-96 h of transfection a satisfactory level of green fluorescence was observed in the pI-EGFP transfected High Five cells indicating a transfection efficiency of around 35-40% (Supplementary Fig. 2).

CSFV *E2* protein was expressed in High Five cells using a laboratory-modified expression vector with a secretory signal peptide at N-terminus, viz. pI-SP-E2 vector. *E2* gene expression in the form of the recombinant secretory protein was detected in the High Five cell culture supernatants collected at 72 h by SDS-PAGE to detect the optimum level of protein expression.

The transfected High Five cells started dying 5-6 days post Zeocin selection and maximum cell death of around 60-70% was observed till 14 days. The Express Five culture medium was replaced every 3rd day and fresh Zeocin antibiotic was added @150 µg/ml medium till 5th week post transfection. The remaining live High Five cells started to grow and multiply slowly from the 4th week onward and the adherent cells (75cc) and suspension culture of High Five-*E2* cells (250 ml) were sub-cultured at the 6th week after observing their appreciable cell numbers. Then (6th week onward) gradually the Zeocin concentration was reduced to 50 µg/ml medium for better growth of the selected High Five cells. Some portions of the stable selected High Five cells were cryopreserved for future use and the rest portions were cultured continuously for harvesting the recombinant secretory *E2* protein. The transformed High Five cells were then adapted to grow in suspension culture in a sterile 250 ml screw capped conical flask at 26°C in a shaker incubator with 60-80 rpm (Fig. 2).

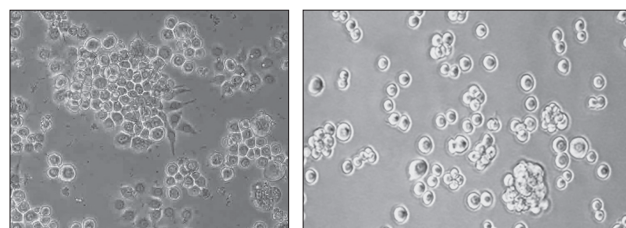


Fig. 2. Different stages of the selection of CSFV *E2* transfected High Five cells.

The purified and concentrated, High Five cell expressed CSFV *E2* secretory recombinant protein was subjected to SDS-PAGE analysis in 12% polyacrylamide gel where a dark and distinct protein band with a molecular weight of ~65 kDa was observed (Fig. 3). The SDS-PAGE detected ~65 kDa protein was confirmed as recombinant *E2* protein in dot blot and western blot analysis by using CSFV anti-sera antibodies which showed good reactivity with *E2* protein. Dot blot assay was carried out by spotting 2-3 µl of the concentrated *E2* protein into the nitrocellulose membrane (NCM) comb along with some negative controls like PBS or fresh cell culture medium. Blots were developed by using different primary and secondary antibodies. Primary antibodies like Classical swine fever virus (CSFV) antisera

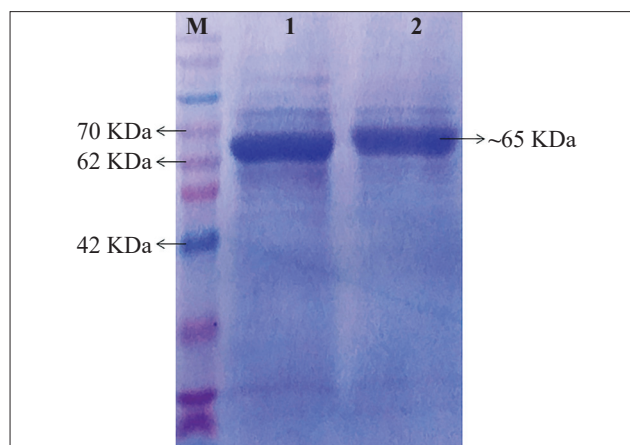


Fig. 3. SDS-PAGE analysis of purified CSFVE2 protein [Lane M: Protein ladder (Cat. No. PG500-0500P1); Lane 1,2: Purified protein].

(at 1:25 dilution) and monoclonal-CSFV antibody (at 1:200 dilution) and secondary antibodies like anti-pig IgG-HRP (1:2000 dilution) and anti-mouse IgG-HRP (at 1:4000 dilution) were used, respectively. A bright brown colored spot appeared on the site where CSFV *E2* protein was spotted on the NCM comb while no color appeared on the negative control sites (Supplementary Fig. 3).

Both dot blot and western blot assays confirmed the expressed protein as CSFV *E2* recombinant protein. The recombinant *E2* protein showed very good reactivity with the CSFV-antisera and slightly less reactivity with MAb against *E2* indicating its diagnostic potential. Madera *et al.* (2016) found that *E2* homodimers have higher affinity to *E2*-specific mAb WH211 than do the monomers, indicating that oligomerization and glycosylation of *E2* protein are important for the induction of protective immune response and neutralizing antibodies. Hua *et al.* (2014) generated a mammalian cell clone (BCSFV-*E2*) that stably produces a secreted form of CSFV *E2* protein (mE2). BCSFV *E2* cells express *E2* protein with high efficiency.

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SUMMARY

Classical swine fever is one of the most important viral diseases of domestic pigs and wild boar. It is a notifiable disease to the World Organization for Animal Health. In the present study, codon optimized CSFV *E2* gene was cloned into a laboratory-modified insect cell expression vector for producing the recombinant protein in secretory form. Endotoxin-free recombinant plasmid prepared from the positive clone was transfected into the High Five insect cell line and then selected against zeocin antibiotic for the development of a stable cell line. The stable High Five cell line successfully secreted *E2* recombinant protein with a molecular weight of ~65 kDa as revealed by SDS-PAGE

and Western blot. The recombinant *E2* protein showed efficient reactivity with known CSFV anti-serum and monoclonal antibodies (MAbs against *E2*) in dot blot assay indicating its potential as a diagnostic antigen.

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