



Expression of Immunodominant Regions of E2 from an Indian Isolate of Classical Swine Fever Virus

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ABSTRACT

Classical swine fever (CSF) is an economically important contagious fatal disease of domestic pigs and wild boars. This disease is caused by classical swine fever virus (CSFV), a member of the *Pestivirus* genus within the *Flaviviridae* family. Upon infection, three proteins of CSFV, namely E2, Erns and NS3 induce detectable antibodies. Since E2 is a major glycoprotein that produces neutralizing antibodies and provides protective immunity, it is widely used as a marker for measuring vaccine efficacy and antibody titer. In the present study, immunodominant regions of E2 glycoprotein from Indian field isolate of CSFV were expressed in *E. coli*. The 336 amino acid long N-terminal ectodomain (full-E2) and the 207 amino acid (aa 173-380) long C-terminal immunodominant region (partial-E2) were expressed and purified as 54kDa and 24kDa recombinant proteins, respectively. Both full-E2 and partial-E2 recombinant proteins were also characterized using MALDI-TOF-TOF analysis as CSFV-E2 structural proteins. The yield of purified full-E2 and partial-E2 recombinant protein was 26 mg and 84 mg/ liter culture, respectively. For raising hyperimmune sera against these proteins, chickens and rabbits were immunized with 200µg purified protein and boosted three times with 100µg purified protein intramuscularly. The sera collected one week after last booster were analyzed for CSFV-specific IgG antibody response using purified CSFV as coating antigen in ELISA. The results revealed that full-E2 induced better antibody response in both rabbits and chicken as compared to partial-E2. This study also indicated that these recombinant E2 proteins can be used as diagnostic antigen in ELISA.

Keywords: CSFV, recombinant, E2, prokaryotic expression, ELISA

Classical swine fever (CSF) also known as hog cholera or pig plague, is an extremely contagious often fatal disease of swine which can spread in an epizootic as well as enzootic form. The causative agent of the disease is a small enveloped classical swine fever virus (CSFV) belongs to the genus *Pestivirus* family *Flaviviridae* (Wengler *et al.*, 1995) and is closely related antigenically and structurally to other two members of the family i.e. bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) (Simmonds *et al.*, 2012; Chakraborty *et al.*, 2012). Blome *et al.* (2017) has reviewed extensively on virus properties, clinical signs, pathology, epidemiology, pathogenesis, immune responses, diagnosis and vaccination of CSFV. The positive sense single-stranded RNA genome of CSFV encodes a single open reading frame for a 3898 amino

acids long polyprotein flanked by a 5'-nontranslated region (NTR) and a 3'-NTR. The polyprotein is processed by viral and cellular proteases into four structural (C, Erns, E1 and E2) and eight non-structural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins.

Upon infection, three proteins, namely E2, Erns and NS3 induce detectable antibodies but antibodies against E2 and Erns confer virus neutralizing potential and protective immunity (Rumenapf *et al.*, 1991; Konig *et al.*, 1995; de Smit *et al.*, 2001; Zhang *et al.*, 2006). The E2 glycoprotein (373 amino acids, 51-55KDa) possesses two independent antigenic units, B/C and A/D (residues 690-800 and 766-865, respectively) that are identified in the N-terminal half of E2 (van Rijn *et al.*, 1994; van Rijn *et al.*, 1993). E2 glycoprotein-based CSF subunit vaccines are considered



to be safe, effective and have DIVA (differentiating infected from vaccinated animal) potential (Lin *et al.*, 2009; Bouma *et al.*, 2000; Moormann *et al.*, 2000; de Smit, 2000). The envelope glycoprotein E2 is widely used as a marker for measuring vaccine efficacy and antibody titer (Moormann *et al.*, 2000; Reimann *et al.*, 2004, Koenig *et al.*, 2007; Sun *et al.*, 2011).

In the present study, two immunogenic regions of E2 structural glycoprotein from an Indian field isolate of CSFV were expressed in *E. coli* and recombinant proteins were analysed for their immunogenicity by raising hyperimmune sera in rabbits and chicken.

MATERIALS AND METHODS

For amplification of E2 coding sequences the recombinant bacmid containing full-length infectious cDNA clone of an Indian CSFV field isolate CSFV/IVRI/VB-131 was used as template in PCR (Kamboj *et al.*, 2014, 2015). Four healthy rabbits and four healthy chickens were given respective food and water *ad libitum* and used for immunization. All ethical and biosafety clearances have been obtained under Institute Biosafety Committee (IBSC) and Institutional Animal Ethics Committee (IAEC) to carry out the study.

Cloning of full-E2 into pRham N-His SUMO prokaryotic expression vector

The coding sequence of E2 gene from an Indian isolate of CSFV (GenBank Accession No. KM262189.1) was translated into protein and analyzed using Protean software (Lasergene). The 336 amino acid long ectodomain (aa position 35-370) without signal sequence and transmembrane region having high hydrophilicity, antigenic index and surface probability was selected for prokaryotic expression as full-E2 protein. The coding sequence for 336 aa full-E2 was amplified from recombinant bacmid (Kamboj *et al.*, 2014, 2015) using PCR using SUMO-E2-Forward 5'-CGCGAACAGATTGCAGGTGGATCCCTAGCCTGCAAGGAAGATTACAGG-3' and SUMO-E2-Reverse 5'-GTGGCGCCGCTCTATTAATCTGAGTGGCGGTCTGTCAC-3' primers. The PCR reaction was carried out in a thermal cycler (Applied Biosystems) in a 20 µl reaction volume comprising 10 µl of 2x Phusion master mix (NEB), 1 µl each of SUMO-E2-Forward and SUMO-E2-Reverse primers and 1 µl

of CSFV-bacmid template. Amplification condition was initial denaturation at 98°C for 30 sec, 30 cycles of denaturation at 98°C for 10 sec and annealing-extension at 55°C for 30sec. The 1008 bp amplified product was cloned into pRham N-His SUMO vector ((Lucigen, USA) by homologous recombination, transformed in *E. coli* competent cells and plated on LB agar plate containing Kanamycin (30 µg/ml) and incubated at 37°C for overnight following manufacturer's instructions. A single colony was grown in 5 ml of LB broth containing 30µg/ml Kanamycin overnight in shaker incubator at 250 rpm, 37°C and recombinant pSUMO-E2 plasmid was isolated by using Sure-spin mini kit (Nucleo-pore) following manufacturer's instructions. The isolated recombinant pSUMO-E2 plasmid was characterized in PCR using E2 specific SUMO-Forward and SUMO-reverse primers.

Cloning of partial-E2 into pET-302 prokaryotic expression vector

The partial-E2 protein expression, 624 bp coding sequence for 173-380 aa from C-terminal region of E2 having high hydrophilicity, antigenic index and surface probability (analyzed using Protean software) was amplified in PCR and 624 bp PCR product was cloned into pET-302 prokaryotic expression vector between *EcoRI* and *XhoI* sites. The recombinant pET-302 plasmid with partial-E2 (pET-partial-E2) was inoculated in 5 ml LB containing Ampicillin (100µg/ml) and grown overnight in shaker incubator at 200 rpm at 37°C. The pET-partial-E2 plasmids were purified using QIA prep spin miniprep kit (Qiagen) following manufacturer's instructions and characterized for the presence of 624 bp E2 insert by nucleotide sequencing.

Expression of full-E2 and partial-E2 recombinant proteins

For expression of full-E2 protein, 50-100 ng of pSUMO-E2 plasmid was transformed in *E. coli* Rosetta-gami 2(DE3) cells and plated on LB agar plate containing 50µg/ml kanamycin and 34µg/ml chloramphenicol and incubated at 37°C overnight (Sambrook and Russell, 2016). A single bacterial colony was inoculated in 5 ml of LB broth containing kanamycin (50µg/ml) and chloramphenicol (34µg/ml) and incubated for 1.5 h in shaker incubator at 200 rpm and 37°C. For induction of protein, 100 µl

(1:100) of overnight grown culture was inoculated in 10 ml LB broth containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol and incubated at 200 rpm, 37°C. When the OD of culture reached to 0.7 after 5 h post incubation, 1 ml of un-induced culture was taken out and cells were pelleted by centrifugation at 5000 × g for 3 min and stored at -20°C. Remaining cells were induced by adding 0.2% L-Rhamnose for 6 h and overnight by incubating in shaker incubator at 37°C and 200 rpm. 1 ml each of 6 h and overnight induced culture cells were pelleted by centrifugation at 5000 × g and stored at -20°C for further use. For characterization of expression after induction, 100 µl of 2X sample loading buffer was added to both un-induced and induced thawed cell pellets and boiled for 10 min. Thereafter, 40 µl of cell lysate along with pre-stained protein molecular weight marker was loaded in 12% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970). The presence of 54 kDa protein in the induced culture indicated expression of full-E2.

For expression of partial-E2 protein, 100 µl (1:100) of overnight grown pET-partial-E2 culture was inoculated in tube containing 10 ml LB broth with Ampicillin (100 µg/ml) and Chloramphenicol (34 µg/ml) and incubated in shaker incubator at 200 rpm and 37°C. When the OD₆₀₀ nm reached at 0.7 after 5 h incubation, 1 ml of un-induced culture was collected and centrifuged at 5000 × g, supernatant was discarded and pellet was stored at -20°C. The remaining culture was induced by adding 1 mM IPTG (from 100 mM IPTG stock) and further incubated for 4 h and overnight. 1 ml of induced cells were pelleted at these time points and stored at -20°C. For characterization of expression, 100 µl of 2X sample loading buffer was added in un-induced and induced cell pellets and boiled for 10 min. Thereafter, 40 µl of cell lysate along with pre-stained protein molecular weight marker was resolved and analyzed in 12% SDS-PAGE. The presence of 27kDa protein in the induced culture indicated expression of partial-E2.

Purification of recombinant proteins

For purification of recombinant proteins, 100 ml culture was induced with 0.2% L-rhamanose and 1 mM IPTG for full-E2 and partial-E2, respectively. The induced cultures were pelleted by centrifugation at 4500 rpm for

30 min and expressed proteins were purified using Ni-NTA chromatography. Briefly, the pellet from 100 ml induced culture was resuspended in 4 ml of lysis buffer (6M Guanidium Hydrochloride, 100 mM NaH₂PO₄, 10 mM Tris-Chloride, pH 8.0) containing 3% SDS followed by incubation for 2 h in orbital shaker at room temperature and shaking with 200 rpm. The cell lysate was centrifuged at 10,000 × g for 30 min at room temperature and supernatant was transferred to a polypropylene column (5 ml capacity) and 1 ml of Ni-NTA agarose resin was added to the lysate followed by the incubation in shaker incubator at 37°C, 200 rpm for 2 h. After incubation, the contents were packed in to a column, the supernatant was allowed to drain and column was washed with 500 µl wash buffer (8M Urea, 100 mM NaH₂PO₄, 10 mM Tris-Chloride, pH 6.3) six times and subsequently washed with 500 µl of another buffer (8M Urea, 100mM NaH₂PO₄, 10 mM Tris-Chloride, pH 5.9) six times. After completion of washing, each protein was eluted with 500 µl of elution buffer (8M Urea, 100 mM NaH₂PO₄, 10 mM Tris-Chloride, pH 4.5) and immediately neutralized by adding 8 µl of 5M NaOH. Thus, 15 elutes of full-E2 and partial-E2 protein were collected and stored at 4°C.

Characterization of recombinant proteins

For characterization of recombinant proteins in SDS-PAGE, 32µl of each elute was resolved in 12% SDS-PAGE gel (Laemmli, 1970) and the presence of expected 54kDa full-E2 and 27kDa partial-E2 protein was checked in gel after staining with commassie blue. For characterization of proteins in Western blot, 32µl each recombinant protein was separated in 12% SDS-PAGE along with pre-stained molecular weight marker. The resolved proteins were subsequently transferred using transfer buffer (50 mM Tris base, 380 mM glycine, 0.1% SDS, 20% methanol) to nitrocellulose membrane (NCM) at current 0.8 mA/cm² of gel for 90 min using a semi-dry blotting apparatus (Bio-Rad). After blotting, the unbound surface on NCM was blocked with 10% horse serum in PBS at 4°C overnight, followed with three washings each for 5 min, with PBS, pH 7.4 containing 0.05% Tween-20 (PBST). The membrane was incubated with CSFV-vaccinated pig serum diluted 1:200 in blocking buffer and incubated at 37°C for 1.5 h in shaker incubator at 80 rpm. The membrane was washed three times with PBST and subsequently incubated at 37°C for 1.5 h with anti-pig IgG-HRPO conjugate (Sigma-

Aldrich) at 1:4000 dilution in PBST. The membrane was washed thrice with PBST and developed by using DAB solution (6 mg of DAB+10µl H₂O₂ in 5 ml with PBS). The reaction was stopped with distilled water and the blot was documented by scanning or in gel documentation system.

Further, the identity of the recombinant E2 proteins was confirmed by mass spectrometry. The purified recombinant full-E2 and partial-E2 were resolved in 12% SDS-PAGE and the respective 54kDa and 27kDa molecular weight proteins were sliced from gel. Peptides obtained after trypsin digestion in the gel were analysed by a MALDI-TOF mass spectrometer at Sandor Life Sciences, Hyderabad, India. Spectra were analysed to identify the protein of interest using Mascot sequence matching software (Matrix Science). Conserved peptide regions identified in mass spectrometry were subjected to Blast analysis in the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The affinity purified partial-E2 (7.5 µl) and the affinity purified full-E2 (14 µl) along with BSA standards (8 to 1µg) were resolved independently in 12% SDS-PAGE. The concentration of each protein was calculated with respect to BSA standards by densitometry in gel documentation system (BioRad).

Immunization of recombinant proteins for raising hyperimmune sera

Two healthy rabbits and chickens in each group were immunized intramuscularly with 200 µg of full-E2 and partial-E2 and boosted three times with 100 µg of antigen at 14, 21 and 28th day post immunization. The sera were collected one week after the last booster dose and stored at -20°C for further use.

Indirect ELISA

Indirect ELISA was performed to analyse the CSFV-specific IgG antibody response in hyperimmune sera. Briefly, the flat bottom 96 wells ELISA plates (Maxisorb, Nunc) were coated with 50 µl of purified CSFV as coating antigen with different dilution (10⁻³ to 10⁻⁶ TCID₅₀/well in coating buffer, pH 9.6). The coated plate was incubated overnight at 4°C. Plates were washed three times after each step with PBST (PBS, pH 7.4; Tween-20, 0.05%) in automated plate washer (Awareness Technology). Blocking

was done with 10% healthy goat serum in PBS 100µl/well and plate was incubated in orbital shaker incubator at 80 rpm, 37°C for 1h. After washing, different dilutions (1:100 to 1:800 in 5% NGS in PBST) of hyperimmune sera (as primary antibody) were added and the plate was incubated for 1 h. After washing, respective secondary antibody (anti-rabbit-HRPO or anti-chicken-HRPO) 50 µl/well of 1:6000 (diluted in 5% NGS in PBST) was added and plate was incubated for 1h. After washing, the reaction was developed by adding 50 µl/well TMB substrate followed by 15 min incubation at room temperature and stopped by adding 50 µl/well 5% Sulphuric acid. The absorbance at 450nm with reference reduction at 630nm was recorded in ELISA reader (Awareness Technology). The data was analyzed by calculating the P/N ratio (Positive-Negative ratio) by taking hyperimmune serum as positive and pre-immune serum as negative.

RESULTS AND DISCUSSION

For E2 ectodomain expression, the signal sequence and transmembrane region of 370 aa long full-length E2 was analysed using Protean software (Lasergene) and 336 amino acid region (35-370 aa) without signal sequence and transmembrane region was selected for prokaryotic expression (Fig. 1).

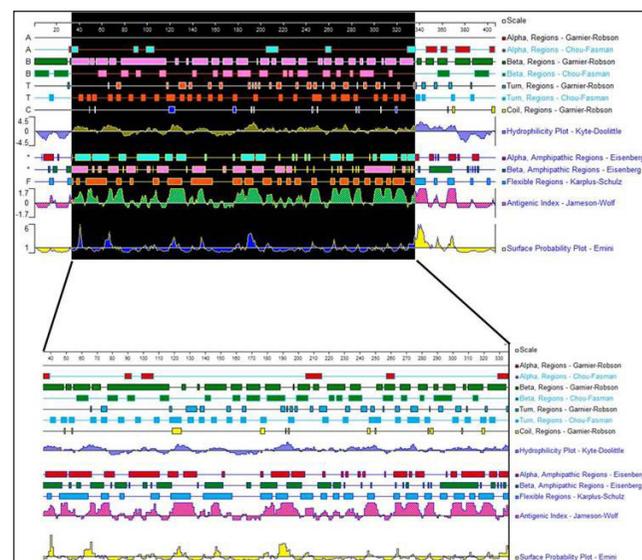


Fig. 1: Amino acid sequence analysis and protein secondary structure prediction of 336 amino acids long N-terminal ectodomain of E2 glycoprotein of CSFV using Protean software (Lasergene).

The coding sequences for 336 amino acid ectodomain was amplified from recombinant bacmid having complete genome from an Indian isolate of CSFV using CSFV-E2 specific SUMO-Forward and SUMO-Reverse primers. The 1008 bp amplified PCR product was characterized in agarose gel (Fig.2 A) and was cloned into pRham N-His SUMO prokaryotic expression vector. The recombinant plasmid (pSUMO-E2) was characterized in PCR using CSFV-E2 specific primers that confirmed the presence of 1008 bp product corresponding to the sequence of CSFV-E2 (Fig.2 B).

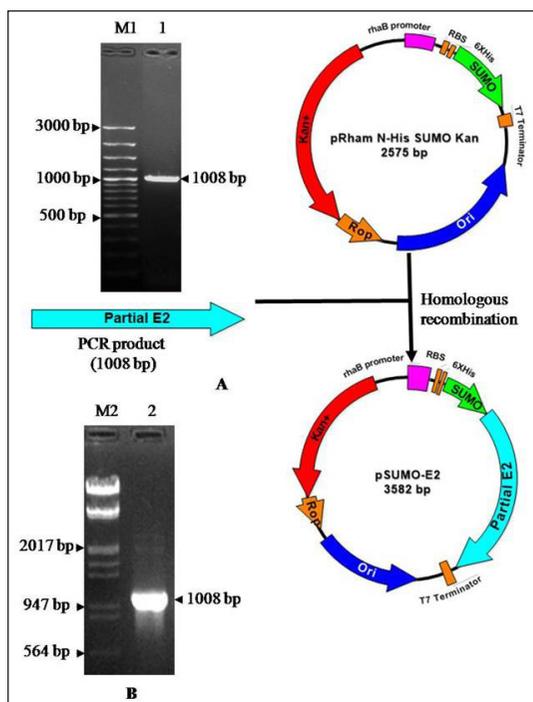


Fig. 2: Cloning of 1008 bp gene fragment encoding 336 amino acid ectodomain (from aa 35-370) of E2 protein into pRham N-His SUMO prokaryotic expression vector. A. Schematic representation of cloning of 1008 bp E2 gene fragment of into pRham N-His SUMO prokaryotic expression vector with N-terminal His and SUMO-tag. The 1008 bp PCR fragment was amplified from CSFV Bacmid and cloned into pRham N-His SUMO prokaryotic expression vector. M1: Gene ruler100bp plus DNA ladder; Lane 1: 1008 bp PCR amplified fragment. B. PCR characterization of recombinant pSUMO-E2 using gene-specific primers. M2: λ DNA/*EcoRI*+*HindIII* DNA marker. Lane 1: 1008 bp PCR amplified fragment.

The recombinant plasmid pSUMO-E2 was transformed into *E.coli* Rosetta-gami 2 (DE3) competent cells. For expression of recombinant protein, transformed cells

were induced with 0.2% L-rhamnose for different time intervals. The expressed recombinant protein Sumo-E2 was analyzed in SDS-PAGE by using collected bacterial cell pellets from un-induced (Fig.3A, Lane 1), and induced cultures for 6h (Fig.3A, Lane 2) and overnight (Fig.3A, Lane 3). The expressed recombinant full-E2 in induced cells was 54kDa. This protein was purified under denaturing conditions by using Ni-NTA affinity column chromatography and analysed in 12% SDS-PAGE as single major band of 54 kDa (Fig.3B, Lane 4) which reacted with CSFV-specific hyperimmune serum in Western blot (Fig.3B, Lane 5). Further, this purified recombinant 54 kDa protein separated in SDS-PAGE was sliced and analyzed in MALDI-TOF-TOF which confirmed the authenticity of CSFV-E2 structural protein (Fig.3C).

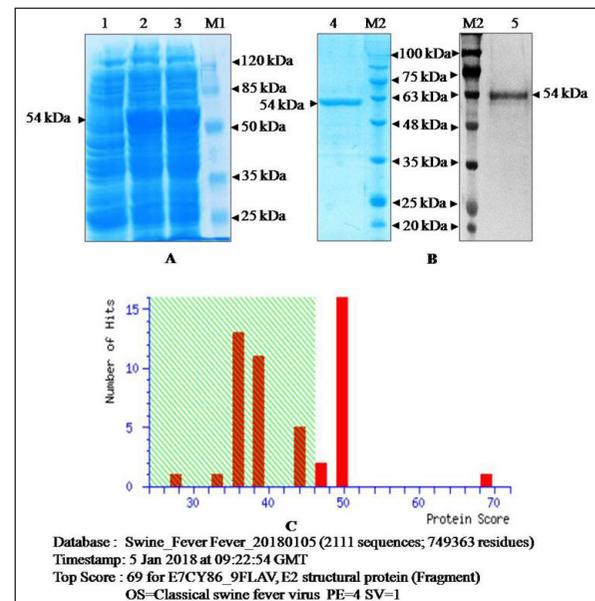


Fig. 3: Prokaryotic expression of 336 amino acid ectodomain of E2 protein with N-terminal His and SUMO tag using pRham N-His SUMO prokaryotic expression vector. A. Recombinant pSUMO-E2 was transformed into *E. coli* Rosetta-gami 2 (DE3) and expression of recombinant protein was induced with 0.2% L-rhamnose. The expressed recombinant protein was detected in SDS-PAGE of bacterial cell pellet collected at 6 h (Lane 2) and overnight (Lane 3) post-induction and compared with un-induced bacterial cell pellet (Lane 1). M1: Unstained protein molecular weight marker. B. The expressed recombinant protein was affinity purified using Ni-NTA and purified recombinant full-E2 protein was analysed in 10% SDS-PAGE (Lane 4) and Western blot (Lane 5) using CSFV-specific hyperimmune serum. M2: Pre-stained protein molecular weight marker. C. MALDI TOF-TOF analysis of the purified recombinant SumoE2 protein.

The purified recombinant full-E2 protein was resolved in SDS-PAGE along with different concentrations (1 to 8 µg) of BSA standard and the quantity of recombinant protein was calculated with respect to standards with the help of densitometer. Densitometry analysis revealed that the concentration of full-E2 protein was 19.2µg/32µl loaded in the gel. The 100 ml culture cells produced 2600 µg purified full-E2 protein thus providing total yield of 26 mg/liter of culture.

For expression of partial-E2, polypeptide region between 173 aa to 380 aa having high hydrophilicity, antigenic index and surface probability was selected (Fig. 4). The 624 bp long coding sequence encoding 207 aa C-terminal immunodominant E2 region was selected for prokaryotic expression as partial-E2. As evident from the strategy depicted in Fig.5A, 624 bp long E2 gene fragment has been cloned in *EcoRI* and *XhoI* sites of pET-302 prokaryotic expression vector. The characterization of recombinant plasmid pET-partial-E2 was done by nucleotide sequence analysis that revealed the presence of E2 gene sequence in between *EcoRI* and *XhoI* sites (Fig.5B).

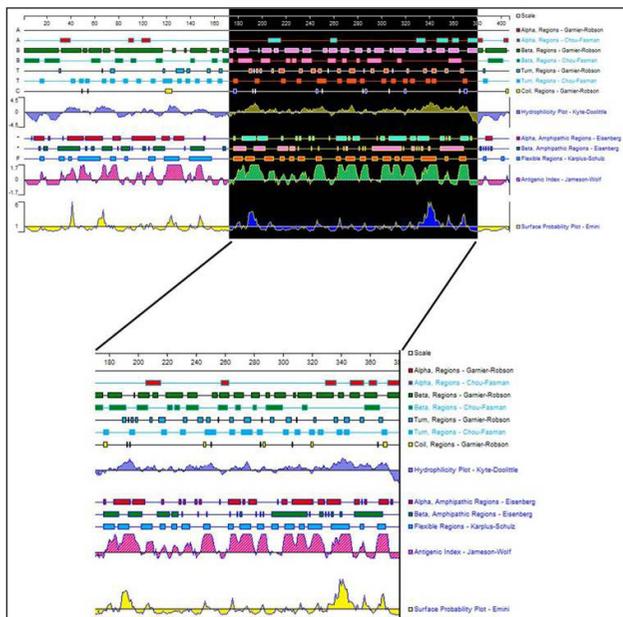


Fig. 4: Amino acid sequence analysis and protein secondary structure prediction of 207 amino acids long C-terminal region of E2 glycoprotein of CSFV using Protean software (Lasergene).

The recombinant pET-partial E2 plasmid was transformed into *E. coli* BL21 (DE3)pLysS competent cells and induced

with 1% IPTG for 4 hrs and overnight. The proteins were separated in SDS-PAGE from collected bacterial cell pellets induced for 4 h (Fig. 6A, Lane 2) and overnight (Fig. 6A, Lane 3) post-induction and compared with un-induced bacterial cell pellet (Fig. 6A, Lane 1).

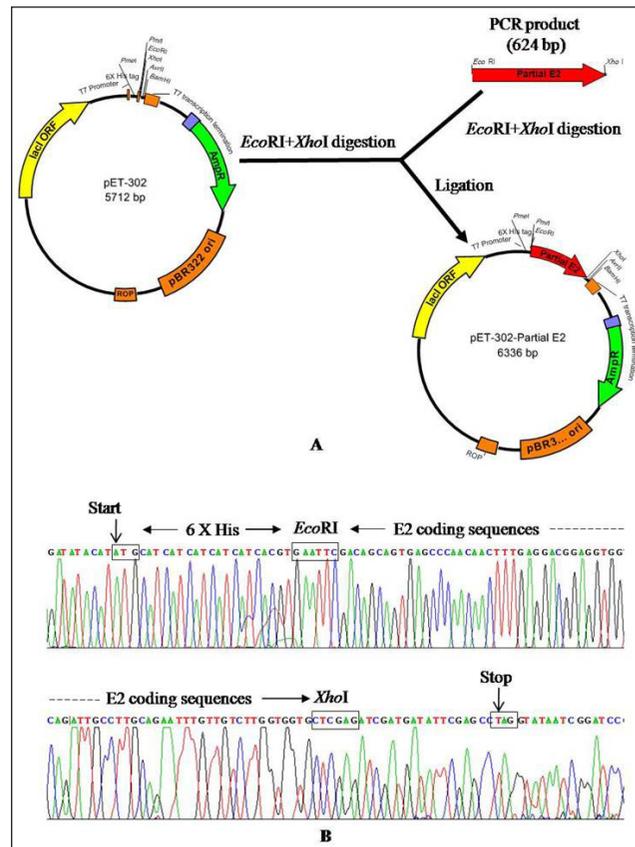


Fig. 5: Cloning of 624 bp gene fragment encoding 208 amino acid C-terminal immunodominant region (from aa 173-380) of E2 protein into pET-302 prokaryotic expression vector. A. Schematic representation of cloning of 624 bp E2 gene fragment of into pET-302 prokaryotic expression vector between *EcoRI* and *XhoI* sites with N-terminal His-tag. B. Characterization of recombinant pET-partial E2 by nucleotide sequencing.

The expression of 27 kDa recombinant protein was observed in induced cultures as compared to un-induced control cells. The recombinant partial-E2 protein was purified through affinity matrix using Ni-NTA chromatography under denaturing condition. Purified recombinant protein was resolved in SDS-PAGE which revealed 27 kDa molecular weight protein (Fig. 6B, Lane 4). The reactivity of this 27 kDa recombinant protein was confirmed in Western blot probed with CSFV-specific hyperimmune serum (Fig. 6B,

Lane 5). The 27 kDa partial-E2 protein, resolved in SDS-PAGE was sliced and analyzed by MALDI-TOF-TOF that confirmed the authenticity of E2 structural protein of CSFV-E2 (Fig. 6C). Densitometry analysis of the purified protein revealed that the concentration of recombinant 27 kDa E2 protein was 19.2 µg/ 32µl loaded in the gel. The 100 ml culture produced 8400 µg purified partial-E2 protein, thus the yield of recombinant partial-E2 protein was 84 mg/liter of culture cell.

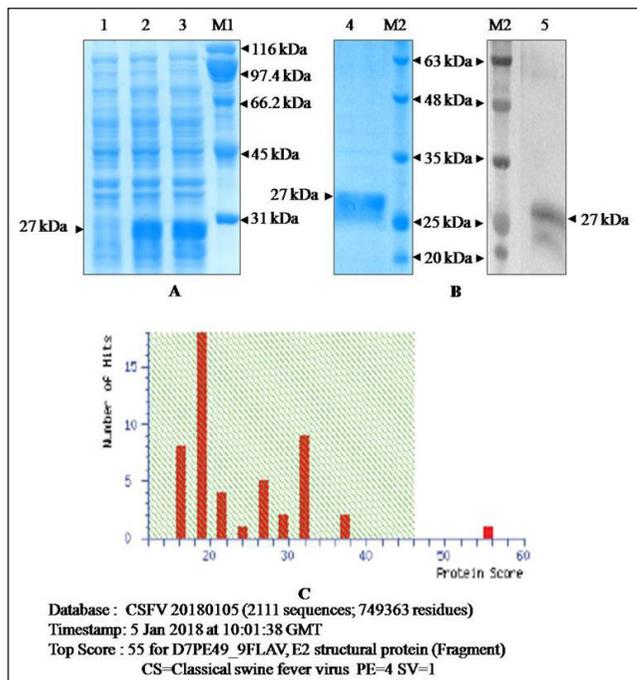


Fig. 6: Prokaryotic expression of 208 amino acid C-terminal region of E2 protein with N-terminal histidine fusion tag using pET-302 prokaryotic expression vector. A. Recombinant pET-partial E2 was transformed into *E. coli* BL21(DE3) pLysS and expression of recombinant protein was induced with 1% IPTG. The expressed recombinant protein was detected in bacterial cell pellet collected at 4 h (Lane 2) and overnight (Lane 3) post-induction and compared with un-induced bacterial cell pellet (Lane 1). M1: Unstained protein molecular weight marker. B. The expressed recombinant protein was affinity purified using Ni-NTA and purified recombinant partial-E2 protein was analysed in 10% SDS-PAGE (Lane 4) and Western blot (Lane 5) using CSFV-specific hyperimmune serum. M2: Pre-stained protein molecular weight marker. C. MALDI TOF-TOF analysis of the purified recombinant partial-E2 protein.

For inducing CSFV-E2-specific antibody, the purified full-E2 and partial-E2 protein were inoculated in rabbit and chickens and sera were collected and analysed in

indirect ELISA. In ELISA, the full-E2-immunized rabbits and chicken yielded P/N ratio >1 and considered sero-positive (Fig.7 A-D). The serum from one rabbit showed higher titer (Fig.7A) than the other (Fig.7B). Similarly, one chicken (Fig.7C) showed higher titer than the other (Fig.7D).

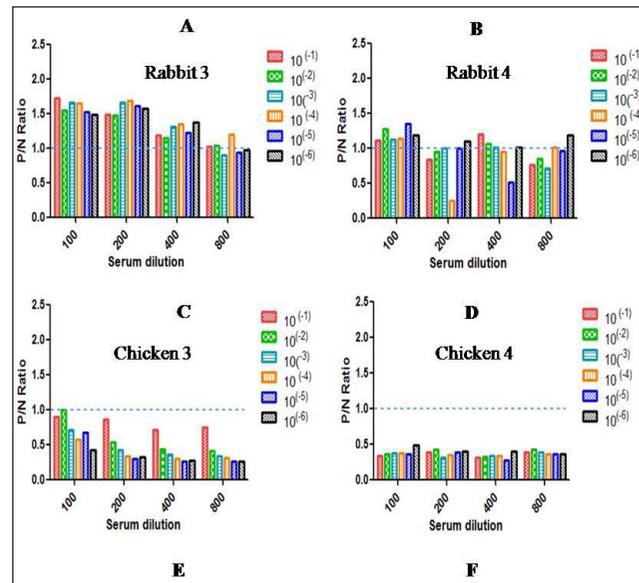


Fig. 7: Evaluation of CSFV-E2 specific IgG response in sera of rabbits (A, B) and chicken (C, D) immunized with full-E2 in ELISA using different dilutions of CSFV as coating antigen.

Likewise, the sera collected from rabbits immunized with partial-E2 reacted and showed sero-positivity against purified CSFV antigen (Fig.8A,B). Among the two rabbits, it was observed that female rabbit (rabbit-1) showed high antibody titer than the male rabbit (rabbit-2). However, both chicken immunized with partial-E2 (Fig.8C, D) revealed P/N ratio <1 and considered sero-negative and incidentally both of these birds were also male.

Various expression systems have been employed for expression of E2 protein, each having its own merits and limitations (Krol *et al.*, 2014; Xia *et al.*, 2014). Mature E2 protein (51-55kDa) is the major envelope glycoprotein playing important role in eliciting the immune response during infection (Qi *et al.*, 2009). Cheng *et al.*, (2015) have expressed secretory form of fully glycosylated CSFV E2 in human embryonic kidney cell line and showed that the supernatant can be used as coating antigen.

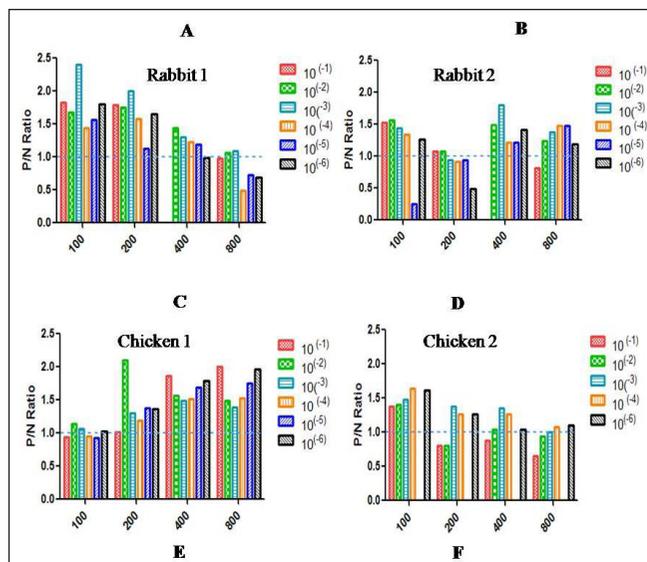


Fig. 8: Evaluation of CSFV-specific IgG response in sera of rabbits (A, B) and chicken (C, D) immunized with partial-E2 in ELISA using different dilutions of CSFV as coating antigen.

Bacterial-expressed recombinant E2 was not found effectively immunogenic due to absence of proper conformation (Andrew *et al.*, 2003), and lack of glycosylation which is required for induction of the optimum neutralizing antibodies against CSFV antigen. Further, prokaryotic expression system has been used for production of recombinant E2 protein in *E. coli* for different purposes such as diagnostic antigen, as immunogen to raise hyperimmune serum etc. For instance, Hua *et al.* (2014) amplified the gene encoding the N-terminal end (340 aa) of the E2 protein from the virulent CSFV Shimen strain using reverse-transcriptase polymerase chain reaction (RT-PCR) and purified the truncated protein expressed in prokaryotic system for the purpose of generating monoclonal antibodies.

It is concluded from the present study that though the yield of prokaryotically expressed partial-E2 (84 mg/liter culture) was found better than that of full-E2 (26 mg/liter culture) yet recombinant full-E2 protein elicited better IgG immune response in both rabbits and chicken as compared to partial-E2.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

REFERENCES

- Andrew, M.E., Morrissy, C.J., Lenghaus, C., Oke, P.G., Sproat, K.W., Hodgson, A.L.M., Johnson, M.A. and Coupar, B.E.H. 2003. Protection of pigs against classical swine fever with DNA-delivered gp55. *Vaccine*, **18**: 1932–1938.
- Blome, S., Meindl-Bohmer, A., Loeffen, W. Thuer, B., and Moennig V. 2006. Assessment of classical swine fever diagnostics and vaccine performance. *Rev. Sci. Tech.*, **25**: 1025–38.
- Blome, S., Moß, C., Reimann, I., König, P. and Beer, M. 2017. Classical swine fever vaccines—State-of-the-art. *Vet Microbiol.*, **206**: 10-20.
- Bouma, A., de Smit, A.J., de Jong, M.C., de Kluijver, E.P. and Moormann, R.J. 2000. Determination of the onset of the herd-immunity induced by the E2 sub-unit vaccine against classical swine fever virus. *Vaccine*, **18**: 1374–1381.
- Chakraborty, S. and Choudhury, S. 2012. Recent trends in the diagnosis of classical swine fever. *Adv. Trop. Med. Public Health Int.*, **2**: 61-71.
- Cheng, T.C., Pan, C.H., Chen, C.S., Chuang, K.H., Chuang, C.H., Huang, C.C., Chu, Y.Y., Yang, Y.C., Chu, P.Y., Kao, C.H., Hsieh, Y.C., and Cheng, T.L. 2015. Direct coating of culture medium from cells secreting classical swine fever virus E2 antigen on ELISA plates for detection of E2-specific antibodies. *Vet. J.*, **205**: 107-109.
- Chung, C.T., Niamela, S.L. and Miller, R.H. 1989. One step preparation of competent *Escherichia coli* transformation and storage of bacterial cells in the same solution. *Proc. Natl. Sci. USA.*, **86**: 2172-2175.
- de Smit, A.J., Bouma, A., de Kluijver, E.P., Terpstra, C. and Moormann, R.J. 2000. Prevention of transplacental transmission of moderate-virulent classical swine fever virus after single or double vaccination with an E2 subunit vaccine. *Vet. Q.*, **22**: 150-153.
- de Smit, A.J., Bouma, A., Van Gennip, H.G., de Kluijver, E.P. and Moormann R.J. 2001. Chimeric (marker) C-strain

- viruses induce clinical protection against virulent classical swine fever virus (CSFV) and reduce transmission of CSF between vaccinated pigs. *Vaccine*, **19**: 1467-1476.
- Edwards, S., Fukusho, A., Lefevre, P.C., Lipowski, A., Pejsak, Z., Roehe, P. and Westergaard, J. 2000. Classical swine fever: the global situation. *Vet. Microbiol.*, **73**: 103-119.
- Hua, R-H., Huo, H., Li, Y-N., Xue, Y., Wang, X-L., Guo, L-P., Zhou, B., Song, Y. and Bu, Z-G. 2014. Generation and efficacy evaluation of recombinant Classical Swine Fever Virus E2 glycoprotein expressed in stable transgenic mammalian cell line. *PLoS ONE*, **9**: e106891. doi:10.1371/journal.pone.0106891
- Kamboj, A., Patel, C.L., Chaturvedi, V.K., Saini, M. and Gupta, P.K. 2014. Complete genome sequence of an Indian field isolate of classical swine fever virus belonging to subgenotype 1.1. *Genome Announc.*, **2**: e00886–e914.
- Kamboj, A., Saini, M., Rajan, L.S., Patel, C.L., Chaturvedi, V. and Gupta, P.K. 2015. Construction of infectious cDNA clone derived from a classical swine fever virus field isolate in BAC vector using in vitro overlap extension PCR and recombination. *J. Virol. Methods*, **226**: 60-66.
- Koenig, P., Lange, E., Reimann, I. and Beer, M. 2007. CP7_E2alf: a safe and efficient marker vaccine strain for oral immunisation of wild boar against classical swine fever virus (CSFV). *Vaccine*, **25**: 3391–3399.
- Konig, M., Lengsfeld, T., Pauly, T., Stark, R. and Thiel, H.J. 1995. Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins. *J. Virol.*, **69**: 6479-6486.
- Krol, E., Pastuch-Gawolek, G., Nidzworski, D., Rychlowski, M., Szeja, W., Gryniewicz, G. and Szewczyk, B. 2014. Synthesis and antiviral activity of a novel glycosyl sulfoxide against classical swine fever virus. *Bioorg. Med. Chem.*, **22**: 2662–2670.
- Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680–685.
- Leifer, I., Ruggli, N. and Blome, S. 2013. Approaches to define the viral genetic basis of classical swine fever virus virulence. *Virology*, **438**: 51-55.
- Lin, G.J., Deng, M.C., Chen, Z.W., Liu, T.Y., Wu, C.W., Cheng, C.Y., Chien, M.S. and Huang, C. 2012. Yeast expressed classical swine fever E2 subunit vaccine candidate provides complete protection against lethal challenge infection and prevents horizontal virus transmission. *Vaccine*, **30**: 2336–2341.
- Lin, G.J., Liu, T.Y., Tseng, Y.Y., Chen, Z.W., You, C.C., Hsuan, S.L., Chien, M.S. and Huang, C. 2009. Yeast-expressed classical swine fever virus glycoprotein E2 induces a protective immune response. *Vet. Microbiol.*, **139**: 369–374.
- Moormann, R.J., Bouma, A., Kramps, J.A., Terpstra, C. and de Smit, H.J. 2000. Development of a classical swine fever subunit marker vaccine and companion diagnostic test. *Vet. Microbiol.*, **73**: 209–219.
- Qi, Y., Zhang, B.Q., Shen, Z. and Chen, Y.H. 2009. Candidate Vaccine Focused on a Classical Swine Fever Virus Epitope Induced Antibodies with Neutralizing Activity. *Viral Immunol.*, **22**: 205-213.
- Reimann, I., Depner, K., Trapp, S. and Beer, M. 2004. An avirulent chimeric Pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus. *Virology*, **322**: 143-157.
- Rumenapf, T., Stark, R., Meyers, G. and Thiel, H.J. 1991. Structural proteins of hog cholera virus expressed by vaccinia virus: further characterization and induction of protective immunity. *J. Virol.*, **65**: 589–597.
- Sambrook, J. and Russell, D.W. 2016. Molecular Cloning: A laboratory Manual, 3rd Edn. Cold Spring Harbor Press, Cold Spring Harbor, New York.
- Sarma, D.K., Krishna, L. and Mishri, J. 2008. Classical swine fever in pigs and its status in India: A review. *Indian J. Anim. Sci.*, **78**: 1311-1317.
- Simmonds, P. 2012. Family Flaviviridae. In: Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses (Eds. Andrew M.Q. King, Michael J. Adams, Eric B. Carstens, and Elliot J.) Lefkowitz. San Diego, CA: Academic Press. pp1003-1020.
- Sun, Y., Li, H.Y., Tian, Q.Y., Zhang, X., Li, N. and Qiu, H.J.D.Y. 2011. A novel alphavirus replicon-vectored vaccine delivered by adenovirus induces sterile immunity against classical swine fever. *Vaccine*, **29**: 8364–8372.
- Van Rijn, P., Miedema, G., Wensvoort, G., Van Gennip, H. and Moormann, R.J. 1994. Antigenic structure of envelope glycoprotein E1 of hog cholera virus. *J. Virol.*, **68**: 3934–3942.
- Van Rijn, P., Van Gennip, H., De Meijer, E. and Moormann, R. 1993. Epitope mapping of envelope glycoprotein E1 of hog cholera virus strain Brescia. *J. Gen. Virol.*, **74**: 2053–2060.
- Wengler, G., Bradley, D.W., Collett, M.S., Heinz, F.X., Schlesinger, R.W. and Strauss, J.H. 1995. Family Flaviviridae. In Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses. pp 415–427.
- Xia, H., Harimoorthy, R., Vijayaraghavan, B., Blome, S., Widen, F., Beer, M., Belak, S. and Liu, L. 2014. Differentiation of classical swine fever virus infection from CP7_E2alf marker vaccination by a multiplex microsphere immunoassay. *Clin. Vaccine Immunol.*, **22**: 65–71.
- Zhang, F., Yu, M., Weiland, E., Morrissy, C., Zhang, N., Westbury, H. and Wang, L.F. 2006. Characterization of epitopes for neutralizing monoclonal antibodies to classical swine fever virus E2 and Erns using phage-displayed random peptide library. *Arch. Virol.*, **151**: 37-54.

