Prokaryotic Expression and Purification of Highly Soluble Partial Glycoprotein Erns of Indian Strain of Classical Swine Fever Virus

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Abstract Classical swine fever (CSF) or hog cholera, caused by a positive stranded RNA virus belonging to the genus *Pestivirus* of the *Flaviviridae* family, is highly contagious and fatal disease of pigs. We report the novel design of construct for production of highly soluble glycoprotein Erns fragment using prokaryotic expression system. A truncated fragment of the *Erns* gene (coding for aa 109–170) denoted as ‘Erns-Ag’ was subcloned and expressed as hexa-histidine tag fusion on both terminus of protein in *Escherichia coli*. The highly soluble recombinant Erns-Ag protein with purity >95 % was purified by one step Ni–NTA affinity chromatography under native condition. Anti Erns-Ag polyclonal antibodies raised in guinea pig was found to react with CSFV antigen in infected MDCK cell line during immunoperoxidase test. The described methodology of producing a highly soluble recombinant protein with native conformation would likely to assist in development of differential diagnostic test as well as its application in raising hyperimmune sera for detection of CSFV antigen either in tissue materials or infected cell lines.

Keywords Classical swine fever virus · Erns · Expression · IPT · Purification

Classical swine fever virus (CSFV), known to cause highly contagious and fatal disease in pigs, belong to the genus *Pestivirus* of the *Flaviviridae* family [24]. The disease occurs in acute, chronic, or subclinical forms and is mainly characterized by fever, leukopenia and hemorrhage, coupled with substantial economic losses to pig industry [14]. CSFV is an enveloped positive stranded RNA virus having 12.5 kb genome containing a single large open reading frame encoding a polyprotein precursor of approximately 4,000 amino acids (aa), which post-translationally cleaves into structural proteins (C, Erns, E1, and E2) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [18]. Neutralizing antibodies against two major glycoproteins; Erns and E2 have been demonstrated previously in infected animals [5, 11, 12, 17, 25]. Glycoprotein E2 has been considered as a candidate antigen to be incorporated in vaccine due to its high immunogenicity [19], whereas, glycoprotein Erns has been considered as potential candidate antigen in development of differential diagnostic test [3, 6].

The envelope glycoprotein Erns (endoribonuclease) is a disulfide-linked homodimer of ~ 100 kDa and known to possess RNase activity as well as binding to many cell types [7]. Structurally, monomeric Erns protein has a size of ~ 45 kDa and contains eight cysteines that form intramolecular disulfide bond. It also lacks a typical transmembrane region and accomplishes its association with the viral envelope via its most-C-terminal region, which most likely forms an amphipathic helix [19]. Previously, attempts were made to express and purify either full length or truncated fragments of glycoprotein Erns in *Escherichia coli*, which resulted in formation of complex protein aggregates, low level of expression, low level binding with Ni–NTA column and subsequent purification under denaturing conditions [8, 9], which

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resulted in loss of native conformation of protein and further, inability to detect such protein under Western blotting. Apart from these, attempts were also made to use synthetic peptides of Erns (37 mer peptide) or synthetic gene, in order to obtain conformational epitopes of Erns and their subsequent use in development of differential diagnostic tests [6, 7, 11]. However, such Erns-ELISA tests were not found to be highly sensitive in detection of CSFV specific antibodies as they used denatured/deformed protein which had lost its epitopes of native conformation. Hence, production of highly soluble native Erns protein either full length or truncated is highly essential in developing a sensitive, specific and reliable differential diagnostic assay.

In India, the high prevalence of CSFV antibodies in pigs belonging to different states of the country suggests that the disease is endemic [13, 20]. Recent classifications of Indian CSFV isolates based on NS5B and 5'-UTR (409 nts) regions indicated the circulation of both genotypes 1 and 2.1 in India [2, 15], with the continued dominance of subgroup 1.1 [16, 21]. Moreover, whole genome sequencing of Indian lapinized CSFV strain has been analyzed [4]. Despite the prevalence of two major Genogroups of CSFV isolates in India, currently, there is no indigenously developed diagnostic assay for isolate differentiation and sero-surveillance of CSFV in Indian pigs. In this study, we describe the novel design of Erns fusion protein; and its over-expression and purification of highly soluble recombinant partial fragment of Erns under native condition following amplification of Erns aa 109–170 region from indigenous CSFV strain (IND/UP-Izt/2009-2/Mukt) and cloning into prokaryotic expression vector (pET32a). This is the preliminary report in the process of developing a diagnostic assay for detection of CSFV antibodies prevalence among pigs in India.

For the current study, a virulent field isolate of Classical Swine Fever virus (IND/UP-Izt/2009-2/Mukt) obtained from the 'CSFV Repository' maintained in the Division of Virology, Indian Veterinary Research Institute (IVRI), Mukteswar, India, was used. For construction and expression of recombinant proteins, pET32a vector (Novagen, USA), *E. coli* TOP10 and *E. coli* BL21(DE3) pLysS cells were used for initial transformation and expression of proteins respectively. A primer set targeting partial region (aa109–170) of Erns protein (denoted as Erns-Ag) of Indian Classical Swine Fever virus (CSFV) isolate was designed based on its recently identified gene sequence (GenBank accession no.GU969033). The construct was designed in such a way that the Erns-Ag region was fused between two hexa-histidine tags (Fig. 1, Panel b). The sequences of the oligonucleotides with added restriction sites for *Nco*I in the forward primer and *Xho*I in the reverse primer at 5' end were as follows:

SERAg For: 5'-gctaCCATGGGCGAGTGTGCCGT GACTTGCAG-3'.

SERAg Rev: 5'-gtgCTCGAGCTCATGATCTCCATA TAAAATGTC-3'.

RNA isolated from CSFV was used as template for preparation of cDNA as per the standard protocol. For amplification of partial fragment of *Erns* gene, the PCR mixture consisted of 50 ng of template, 25 pmol of each primers (SERAg For and SERAg Rev), 10 mM of each dNTPs, 1 × PCR buffer, 2.5 mM MgCl₂, and 1 U of *Taq* DNA polymerase. The volume of reaction mixture was made up to 25 µl with Milli-Q water. PCR was performed with following conditions: Initial denaturation at 94 °C for 6 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 4 min. The PCR amplified (size ~ 207 bp) product (50 µg) was gel eluted and ligated to pET32a prokaryotic expression vector (50 ng/µl) in a 10 µl ligation reaction containing 2 × ligation master mix (Qiagen, Germany). The ligation-reaction mixture was incubated for overnight at 16 °C and subsequently used for transformation. *E. coli* TOP10 cells and BL21(DE3)pLysS cells were used for initial transformation and expression respectively, following use of ampicillin (50 mg/ml) and chloramphenicol (35 mg/ml) antibiotics in LB agar for selection of recombinant clones.

For expression and purification of rErns-Ag protein, recombinant bacterial (*E. coli* BL21(DE3)pLysS) cells harboring recombinant plasmid pErns-Ag was grown in 500 ml Luria–Bertani broth with appropriate antibiotics and kept in shaking incubator at 30 °C before induction with 1 mM IPTG. After 3 hour post induction, cells were harvested by centrifugation at 6,000 rpm for 20 min and cell pellet was resuspended in 20 ml buffer (50 mM Tris–HCl, pH 7.8, 100 mM NaCl and lysozyme) before lysis by sonication (Sonics, USA). The Erns-Ag domain protein was purified by affinity chromatography using Ni–NTA Superflow Cartridges (Qiagen, USA) as per the standard protocol. Following column binding and washing with buffer (50 mM Tris–HCl, pH 6.0, 100 mM NaCl and 50 mM Imidazole), rErns-Ag was eluted with elution buffer (50 mM Tris–HCl, pH 7.8, 100 mM NaCl and 300 mM Imidazole). Eluted rErns-Ag fractions were pooled and dialyzed in a buffer (50 mM Tris–HCl, pH 7.8, 100 mM NaCl) and concentrated (Vivaspin, Germany) before storage at –20 °C for further quantitative and qualitative analysis. SDS-PAGE was performed at constant current of 30 mA in a 10 % acrylamide gels and the proteins were analyzed through staining with Coomassie Brilliant blue. Purified proteins were quantified by using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). The purified recombinant Erns-Ag (~ 100 µg), was used to raise the hyperimmune sera in guinea pig following

Fig. 1 Schematic of Erns-Ag construct, expression and purification. **a** Full length mature glycoprotein Erns (endoribonuclease) of Classical Swine Fever Virus, **b** Truncated glycoprotein Erns-Ag domain of Classical Swine Fever Virus with hexa-histidine tag on both termini (N- and C-), **c** PCR amplified Erns-Ag fragment (Lane-M: DNA standard marker; Lane-1: Amplified Erns-Ag fragment (~ 207 bp); Lane-2: Negative control), **d** Expression and purification of rErns-Ag (Lane-M: Protein standard marker; Lane-1: before

induction-0 h; Lane-2: after 3 h induction with 1 mM IPTG; Lane-3: Purified Erns-Ag using Ni-NTA cartridges; Lane-4: Western blotting using Guinea pig hyperimmune sera raised against rErns-Ag), **e** Immunoperoxidase test (IPT) of CSFV infected MDCK cell line (Inset 1: Healthy MDCK cell line as control; Inset 2: CSFV infected MDCK cell line following IPT using guinea pig hyperimmune sera [Guinea pig anti-rErns-Ag antibodies] and DAB as substrate. Arrow denotes cells with CSFV-antigen [Ag]-antibody [Ab] complex)

the standard procedure. The specific reactivity between protein and hyperimmune sera was analysed by counter-immunoelectrophoresis (CIE) and Western blotting as per the standard procedure. Further, immunoperoxidase test (IPT) was carried out using anti-Erns-Ag polyclonal antibodies raised in guinea pig to assess its ability to detect CSF virus in infected cell line. Briefly, 24 well-plates containing MDCK cell monolayer infected with CSFV (previously adopted to grown in heterologous MDCK cells) was incubated for four day at 37 °C under 5 % CO₂ tension before harvesting on fourth day for staining. The cells were fixed with 2 % paraformaldehyde at 25 °C for 15 min followed by washing thrice with 1 × PBS. Anti-Erns-Ag polyclonal antibodies (1:25 dilution with PBS) were added to each well and incubated at 25 °C for 1 h. Following incubation and washing with 1 × PBS, rabbit anti-guinea pig IgG HRPO conjugate (Dako, Denmark) at 1:300 dilution with PBS was added to each well and incubated at 25 °C for 1 h. Color was developed using 3, 3'-diaminobenzidine at 25 °C. After the development of color, cells were washed with PBS, dried in air and observed under microscope and photographed.

Generally, CSFV isolates from different parts of the world have been placed into various genomic groups/sub-groups based on nucleotide sequence variations in E2, 5'UTR and NS5B [10]. The major Genogroups are 1, 2 and 3 and 10 subgroups. Most of the CSFV isolates made all over the world during 1980 and 1990 were of Genogroup 2. Moreover, in the previous phylogenetic analysis reports [20, 22], some of the Indian isolates of CSF virus (IND Tri-Agt 2009-1 Muk and India LP virus) were placed in Genogroup 1.1 and the recent virulent Indian CSFV isolate (IND/UP-Izt/2009-2/Mukt) had been included under Genogroup 2.2 [unpublished data]. The belonging of recent Indian virulent isolate (IND/UP-Izt/2009-2/Mukt) distinctly different from earlier reported CSFV isolates indicate the probable change in Erns protein sequence and subsequently the virulence as noticed in the recent CSFV outbreak. The deduced amino acids comparison among Indian isolates revealed the percentage identity and divergence ranged between 86.3–91.6 and 8.9–15.1 respectively (data not shown). Among all the Erns sequences of foreign and Indian isolates, a total percentage of identity and divergence were found to be 84.1–100 and 0–17.9

respectively. However, the partial region between aa109–170 showed nearly 90 % homogeneity observed among all three Indian isolates. From a full length Erns region of CSFV (Fig. 1, Panel a), an amplification 1 of partial fragment as per the design (Fig. 1, Panel a) from virulent field isolate (IND/UP-Izt/2009-2/Mukt) from India, gave an amplicon of ~ 207 bp in size (Fig. 1, Panel c, lane 1). We selected virulent CSFV isolate (IND/UP-Izt/2009-2/Mukt), based on the recent analysis of Indian field isolates of CSFV, which were classified mainly into Genogroup 2.2 [1]. pET32a vector was used for construction and expression of rErns-Ag proteins as this vector was found to be suitable for enhancing the solubility of recombinant proteins.

Induction of recombinant cells harboring pErns-Ag plasmid, resulted in over-expression of recombinant truncated glycoprotein Erns-Ag protein along with two hexahistidine tags on its both termini which accounted for a total molecular weight of approximately 25 kDa (Fig. 1, Panel d, lane 2), out of which ~ 9 kD and ~ 16 kDa were belonging to Erns-Ag and coding region of pET32a vector along with his-tags respectively. The N-terminus of Erns-Ag protein had a total of 161 residues including hexahistidine tag, whereas C-terminus also had a total of 8 residues including another hexahistidine tag. The solubility analysis of expressed protein revealed majority (>90 %) in soluble fraction than the insoluble fraction (10 %). The high solubility of protein could be due to fusion protein with two histidine tags. Previously, many researchers have noticed the positive effect of the hydrophilic nature of the histidine tag on enhanced expression and high solubility of expressed recombinant fusion proteins [23]. It is also presumed that the native conformation of Erns-Ag was preserved as it was held up on both sides by additional residues along with histidine regions that could have prevented it from forming a disulfide bridges between two monomer units which might have lead to the formation of either dimer or multimer (complex oligomer). Since, it has been known that Cys171 was involved in Erns dimerization as it forms a disulfide bridge with Cys171 of the other monomer in the Erns dimer [7], we did not include the Cys171 residue in our construct. The purification of Erns-Ag protein under native conditions also produced single band of ~ 25 kDa protein with high purity (>98 %) following a one step affinity chromatography without further need for downstream processing using gel filtration or FPLC. A total yield of >40 mg/litre was obtained under described purification conditions. However, the production could be further enhanced by using fermentors with regulated optimum conditions. Moreover, soluble and native conformation protein was devoid of non-specific proteolytic degradation and aggregations which were noticed by earlier researchers [8, 9], during

purification under denaturing conditions. Further, the purified protein and its ability to raise the specific antibodies in guinea pigs was confirmed by the specific reactivity between the purified Erns-Ag protein and hyperimmune sera by the presence of precipitin during the counter-immunoelectrophoresis (CIE) as well as Western blotting (Fig. 1, Panel d, Lane 4). Moreover, the detection of CSFV in infected MDCK cell line following IPT (Fig. 1, Panel e, Inset 2) indicated the native conformation of purified recombinant protein and its ability to raise specific antibodies which are capable of binding with native full length glycoprotein Erns. Primary antibodies (guinea pig anti-Erns-Ag) could be further used for detection of CSFV antigen using IPT and IFT. Till now, only monoclonal or polyclonal antibodies raised against E2 regions are being used for these tests [26]. This could also be used as an alternate primary antibody in addition to primary antibodies against E2.

Since, the polypeptide (Erns aa 109–160) had been previously described as good antigen candidate for detection of antibodies against the CSFV in pigs [8], we presume that the purified highly soluble recombinant (Erns aa 109–170) protein with high quantity in the current study, would likely to assist in the development of diagnostic assays for clinical and sero-surveillance of CSFV antigen and antibodies among pigs of developing countries including India. This is of imminent importance, since in India, classical swine fever (CSF) outbreaks are generally are controlled by immunization of susceptible pigs with lapinized CSFV vaccine [4, 20] and it is difficult to differentiate the infected from vaccinated due to the fact that most of the pigs are serologically indistinguishable, which warrants the necessary of marker vaccine as well as companion diagnostic assay against CSFV infection in India. Moreover, application of prokaryotic expression system for potential diagnostic antigens has several advantages viz., high level of protein expression, rapid cell growth and simple media requirement coupled with one step purification during downstream processing which substantially reduce the cost of production of diagnostic reagent as well as kits. Conclusively, the study indicated the high level of expression and solubility of rErns-Ag protein due to the presence of hydrophilic histidine tags on both terminus. Hence, we foresee multiple advantages of using recombinant Erns-Ag protein as well as anti-Erns-Ag polyclonal antibodies in Indian scenario. First of all, eventually, if a CSFV Erns deletion mutant live virus vaccine or glycoprotein E2 based subunit vaccine developed in India, the Erns-Ag based ELISA could potentially serve as valuable companion diagnostic test that differentiate between infected and vaccinated animals. Secondly, the guinea pig anti-Erns-Ag polyclonal antibodies could be effectively used in demonstration of CSFV antigen in different

infected tissues/organ materials by immunohistochemistry and/or infected cell lines by IPT as confirmatory tests.

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