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Production of recombinant E^{rns} protein of classical swine fever virus and assessment of its enzymatic activity: A recombinant Newcastle disease virus-based approach

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ABSTRACT

Classical swine fever virus (CSFV) is an etiological agent of classical swine fever. The structure protein E^{rns} of CSFV assists in the initial attachment of the virus to the host cell and its subsequent entry. Newcastle disease virus (NDV) has been used as a virus vector to express different proteins successfully. The unique characters of NDV lying in its broad host spectrum and ease in production using embryonated chicken egg make it a choice of vector for expressing foreign gene. In the present work, we are reporting the characterization of the ribonuclease (RNase) activity of recombinant NDV expressing E^{rns} of CSFV. The RNase activity of viral expressed recombinant E^{rns} was found to be stable at a optimum temperature of 50 °C. The study will be useful to express and characterize an enzymatic protein in its native form using the NDV as a vector.

1. Introduction

Classical swine fever (CSF) is an infectious viral disease of domestic pigs and wild boars. CSF is a global problem however, it is mainly reported from Asia, parts of Africa, Central and South America and Europe [1]. Outbreaks of CSF caused huge economic losses in the Netherlands in 1997–1998 [2]. In the 20th century, several European countries have been affected by CSF outbreaks [3]. The clinical symptoms of CSF vary with age of infected animals and viral virulence [4]. The piglets are more susceptible as compared to adults [5].

The classical swine fever virus (CSFV) belongs to the genus *Pestivirus* under family *Flaviviridae* [6]. CSFV isolates are classified into three genotypes and eleven sub-genotypes based on nucleotide sequences of 5′ UTR (untranslated region), E2 and NS5B genes [7,8]. The genome of CSFV comprises of single-stranded positive-sense RNA of approximately 12.3 kb [9]. The genome of CSFV encodes a single open reading frame (ORF) flanked by 5′ and 3′ UTR. The large polypeptide is processed by cellular and viral proteases to give four structural proteins (C, E^{rns}, E1, E2) and eight nonstructural protein (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) [9]. The core protein (C) forms a complex with the viral RNA [9]. CSFV has three glycosylated proteins, namely, E1, E2, and E^{rns}. The E1 and E2 mediate the CSFV entry inside the host cell [10]. The E1 protein is a type I trans-membrane protein, which helps in the attachment of the virus to host cell [10]. The E2 protein forms a homodimer for the entry of CSFV into the host cell and heterodimer for its attachment [11]. The E2 protein has a conserved antigenic region [12]. The Npro protein has auto-protease (cysteine protease) activity and it has been reported to be an antagonist of dsRNA-mediated apoptosis [13,14]. The E^{rns} of CSFV plays an important role in replication of *pestiviruses* and regulation of its RNA synthesis in infected cells [15]. E^{rns} does not have transmembrane anchor amino acids, which helps it to secrete out from the infected cells [16]. The deletion of E^{rns} in CSFV inhibits its transmission to different host cells [17]. In addition, E^{rns} contains virus neutralization motif such as ^{117}DKN^{119} that inhibits the dsRNA-induced IFN-β production [11,18]. Amino acid sequence comparison of E^{rns} protein of pestiviruses showed homology with active site domain of ribonuclease of the T2 family [19,20]. Non-glycosylated E^{rns} protein shows a reduction in its RNase activity approximately 30–40% as compared to glycosylated E^{rns} [21]. Moreover, the RNase activity of E^{rns} induces the apoptosis in lymphocytes causing immune suppression of the host cell [22].

Reverse genetics of Newcastle disease virus (NDV) has been used to develop viral vectors to expressing the foreign genes for vaccine development [23–25], for gene therapy [26,27] and as an oncolytic agent [28–30]. NDV can be cultured with high titers in embryonated chicken eggs, cell culture and respiratory tract of avian and non-avian species [31]. It elicits both antibody-mediated immune response as well as the cellular immune response in the susceptible host [32]. It lacks DNA

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phase in its life cycle and attracts scientist to explore it as non-integration vector. Recombinant NDV shows high and stable expression of foreign proteins both *in vitro* and *in vivo* with very high functional activity [33]. NDV vectors are reported to accommodate foreign gene up to the size of ~3.8 kb [24,34].

It has been shown that glycosylation of structural proteins is important for CSFV survival inside the host cells [35]. NDV expressed foreign proteins are shown to retain their glycosylation and immunogenic properties [23,36–38]. Although NDV has been explored for the expression of the foreign proteins either for the vaccine study or for its oncolytic activity, the enzymatic activity of its expressed protein has not been explored. In the present study, we have characterized the recombinant E^{rns} of CSFV expressed using NDV as a vector.

2. Material and methods

2.1. Cells and virus

The porcine kidney cells (PK-15) were purchased from ATCC (Manassas, USA). Dulbecco's modified Eagle's medium (DMEM) was used for maintaining the cells with 10% fetal calf serum (Invitrogen, USA) and 1% penicillin and streptomycin antibiotics (Invitrogen, USA) at 37 °C under 5% CO_2. The CSFV vaccine (IVRI Vaccine Lapinized) strain was obtained from the college of veterinary science, Guwahati, India. The PK-15 cells were infected with CSFV using standard protocol [39]. The CSFV particles were recovered by repeated freeze and thaw followed by its filtration through 0.22 μm filter. The titer of CSFV stock was calculated by immunoperoxidase staining using CSFV monoclonal antibody as described earlier [40]. The CSFV stock was stored at −80 °C for future use. The NDV strain LaSota was amplified in 9-day-old specific-pathogen-free (SPF) embryonic chicken eggs. The growth of LaSota was confirmed by HA and RT–PCR. The recombinant vaccinia virus strain Ankara expressing T7 RNA polymerase (a generous gift of Dr. Bernard Moss, NIH) was amplified in primary chicken embryo fibroblast cells (CEF) and stored at −80 °C.

2.2. Construction of recombinant NDV antigenome with CSFV surface glycoprotein E^{rns}

The NDV strain LaSota was used to construct antigenomic cDNA plasmid. The antigenomic sequence was flanked with the T7 promoter and hepatitis delta virus ribozyme sequence. The E^{rns} protein gene of CSFV was cloned in full-length antigenomic cDNA between the non-coding sequence of P and M gene. The E^{rns} protein gene was designed to contain the gene start and gene end sequences of M gene of NDV (Fig. 1). The forward primer 5′A**GGCGCGCC**T*TAAGAAAAAA***T***ACGGG TAGAA**GCCACC**ATG**gaaaatataactcaatggaacct-3′ and reverse primer 5′-A**GGCGCGCC**TTA**ggcataggcaccaaacca-3′ containing *Asc*I sites (boldface), NDV gene end and gene start transcriptional signals (italics and underlined), T is an intergenic sequence (boldface) of gene start and gene end was used to amplify the E^{rns} gene cassette. The extra ATG and TTA sequences were added to use as start codon and stop codon for E^{rns} gene. The Ex Taq DNA polymerase was used to amplify the E^{rns} gene cassette (Takara, Japan) and digested with *Asc*I (NEB, USA) for further cloning. The sequence integrity of the E^{rns} gene was confirmed by sequence analysis. The full-length NDV plasmid bearing the E^{rns} gene was assigned as pNDV-E^{rns}.

2.3. Recovery and characterization of recombinant NDV virus expressing CSFV surface glycoprotein E^{rns}

The recombinant NDV expressing the E^{rns} (rNDV-E^{rns}) was recovered from pNDV-E^{rns} after its transfection with other three accessory plasmids bearing the N, P and L genes of the NDV. Briefly, recombinant vaccinia virus strain Ankara expressing T7 polymerase was used to infect the PK15 cells. Simultaneously, the PK15 cells were transfected with pNDV-E^{rns} (2 μg) and three accessory plasmids containing the NDV genes N (1 μg), P (1 μg) and L (0.5 μg) using transfection reagent (Takara, Japan) following the manufacture's protocol. The rNDV-E^{rns} were plaque purified and amplified in 9-day-old embryonic SPF chicken eggs. The rNDV-E^{rns} were further concentrated by ultracentrifugation using 25% sucrose cushion.

The integrity of E^{rns} gene was confirmed by RT-PCR from genomic RNA isolated from rNDV-E^{rns}. The presence of E^{rns} was confirmed by western blot using polyclonal CSFV antibodies. The presence of NDV was confirmed by the monoclonal antibody against HN protein (a kind gift from Dr. Ron Iorio, University of Massachusetts Medical School). The mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays were performed following OIE procedure [41].

2.4. Prokaryotic expression of E^{rns} protein of CSFV and its characterization

The complete E^{rns} protein gene (GenBank accession number NC002657) was cloned into prokaryotic expression vector pET28a (Novagen, Germany) flanking *BamHI* and *Xho*I restriction sites. The integrity of the E^{rns} gene after cloning was confirmed by sequencing. The pET28a containing complete E^{rns} gene was transformed into *Escherichia coli* BL21 (DE3) pLysS cells (Novagen, Germany) and the expression of E^{rns} was induced by 1 mM isopropyl-β-*D*-thiogalactoside (MBI Fermentas, Germany). The fusion protein was extracted after induction of transformed BL21 (DE3) pLysS cells for 4 h at 37 °C and purified by affinity chromatography containing Ni-NTA (Invitrogen, USA). The histidine-tagged at the N-terminal of pET28a was used to purify the E^{rns} protein by affinity chromatography. The recombinant protein was further purified using the Amicon ultra-4 centrifuge filter device (Millipore, USA) with a membrane cut-off of 10 kDa and further dissolved in PBS with 5% glycerol. The concentration of the purified recombinant E^{rns} (rE^{rns}) protein was determined by modifying Lowry's protein assay kit according to the manufacturer's protocol (Pierce, USA). The expression of the rE^{rns} protein was further confirmed by SDS-PAGE and western blot using an anti CSFV polyclonal antibody (obtained from IVRI, India).

2.5. RNase activity assessment of rNDV-E^{rns} and rE^{rns}

RNase activity of rNDV-E^{rns} and rE^{rns} was investigated on RNA isolated from PK-15 cells. The isolated cellular RNA (1 μg) was incubated with 60 nM of rNDV-E^{rns} in a 15 μl reaction buffer (25 mM Tris-HCl, pH 8.4, 100 mM KCl) at 37 °C for 1 h. The rNDV was used in all the reaction as a negative control. The temporal RNase activity of the rNDV-E^{rns} was performed to determine the optimum time-interval ranging from 15 min up to 2 h in the same reaction buffer. In addition, effects of salt, pH, temperature and metal ions on the RNase activity of rNDV-E^{rns} were also determined. The RNase activity of rNDV-E^{rns} was studied using different salts like NaCl, KCl, and NH_4Cl at varying concentrations of 50, 100, 150 and 200 mM. The optimal pH was determined by substituting the buffer with either 25 mM sodium citrate (pH 4.0–5.0), MES (pH 6.0), Tris-HCl (pH 7.0–8.0), or CAPS (pH 9.0–10.0). The thermo-stability of rNDV-E^{rns} was determined by performing the reaction at temperature gradient from 15 to 100 °C.

For the bacterial expressed recombinant protein, the cellular RNA was incubated with 1.7 μM of rE^{rns} protein in a 15 μl of the above reaction buffer at 37 °C for 1 h. The activity of the rE^{rns} protein was estimated in presence of Mg^{2+} ions. Subsequently, Mg^{2+} was substituted by other metal ions like Mn^{2+}, $Ca^{2+}Ni^{2+}$, and Zn^{2+} ions to know the divalent metal ions dependency of RNase activity. In addition, EDTA (10 mM) was also used to chelate the metal ions in specific reaction condition to assess the metal-dependent activity of rE^{rns}. The effect of various salts (NaCl, KCl, and NH4Cl) of different concentrations (50,100, 150 and 200 mM) on the RNase activity of rE^{rns} was studied. The optimal pH was determined by substituting the buffer with different pH viz. 25 mM sodium citrate (pH 4.0–5.0), MES (pH 6.0), Tris-