Evaluation of immunogenic proteins of classical swine fever virus for possible use for its vaccine and diagnostic

A thesis for

DOCTOR OF PHILOSOPHY

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Under the supervision of

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Dedicated to my Parents and Family

INDIAN INSTITUTE OF TECHNOLOGY

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

STATEMENT

I do here by declare that the research embodied in this thesis is the result of experiments carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Dr. Sachin Kumar.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other research.

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Evaluation of immunogenic proteins of classical swine fever virus for possible use for its vaccine and diagnostic"** by Mr. Rakesh Kumar for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, IITG. The work embodied in this thesis has not been submitted elsewhere for a degree.

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Evaluation of immunogenic proteins of classical swine fever virus for possible use for its vaccine and diagnostic.
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Preface

The written thesis constitutes four and half years of scientific research supported by North Eastern Region Biotechnology Programme Management Cell, India. The experimental work and supervision have been carried out at viral immunology lab of the department of Biosciences and Bioengineering at Indian Institute of Technology Guwahati, India. A ten week-term work at the Veterinary College Khanapara, Guwahati, and Barapani veterinary college was carried out for the purpose of antibody generation and vaccine application. The past 4.5 years had uncountable memorable moments of research and its achievements. Many people have contributed directly, indirectly or peripherally for the Ph.D. to be complete. I can strongly say, with no doubt in mind that, I could not have completed this research work alone. I would like to express my gratitude to a lot of people in this regard. First of all, IITG, department, our laboratory and its environment and hospitality are at the receiving end of my gratitude. The IITG atmosphere will always stay close to my mind, and remind me of a great time in my life. I would like to thank my supervisor Dr. Sachin Kumar for his enthusiastic guidance and readiness for any type of support and for guiding me in keeping track of the overall goals and achievements. All my friends circle, former and current lab members are duly appreciated for their unsarcastic and sometimes sarcastic humor and friendly nature which has been very enjoyable during my Ph.D. time. This is very much appreciated and it has been a pleasure to have worked with you. I would like to thank the research groups at Veterinary College Khanapara, Guwahati, and Barapani veterinary college for their support. Many thanks to my guide and Dr. Monika Koul, for their patience to correct my thesis and research papers. A special thanks to Vishnu, Aparna and Nakul who worked with me for a short time and also thanks to Urmil for drawing replication cycle figure of CSFV.

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In loving memory of my father!

Rakesh Kumar

Abbreviation

aa	Amino acid(s)
Ab	Antibody
BAC	Bacterial Artificial Chromosome
BDV	Border disease virus
bp	Base pair
BVDV	Bovine viral diarrhea virus
Cp	Cytopathogenic
CSF	Classical Swine Fever
CSFV	Classical Swine Fever Virus
DIVA	Differentiating Infected from Vaccinated Animals
DdRp	DNA dependent RNA polymerase
(c)DNA	(complementary) Deoxyribonucleic acid
ds	Double stranded
ER	Endoplasmatic reticulum
FMDV	Foot and mouth disease virus
GFP	Green fluorescent protein
h.p.t/i./e.	Hours post transfection/infection/electroporation
HS	Heparan sulfate
IFN	Interferon
IRES	Internal ribosome entry site
Kan	Kanamycin
Kb	Kilobase
mAb/pAb	Monoclonal/Polyclonal antibody
PMV	Paramyxovirus
Ncp	Non-cytopathogenic
NDV	Newcastle disease virus
NGS	Next generation sequencing
NS/NSP	Non-structural/Non-structural protein
Nt	Nucleotide
NTPase	Nucleoside triphosphatase
OIE	Office Internationale des Epizooties
ORF	Open reading frame
(q)PCR	(quantitative) Polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RdDp	RNA dependent DNA polymerase
RNA	Ribonucleic acid
RT	Reverse transcriptase / room temperature
SP/ase	Signal peptide(ase)
SPP	Signal peptide peptidase
Ss	Single-stranded
UTR	Untranslated region
VRP	Virus replicon particle

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Abstract

Classical swine fever (CSF) is an important viral disease of domestic pigs and wild boar. The causative agent classical swine fever virus (CSFV) is enveloped and contains positive sense RNA genome. It belongs to family Flaviviridae and the genus Pestivirus. The CSFV is very stable in extreme environmental conditions especially in cold frozen meat. The structural proteins E2 and E^{rns} of CSFV are immunogenic and participate in the attachment of the virion to the host cell surface and its subsequent entry. In this study, the molecular and physical characterization of lapinized CSFV vaccine was performed. The bacterial expressed recombinant E2 protein was used to develop diagnostic against CSFV infection in swine.

Newcastle disease virus (NDV) is being used as a viral vector to express heterologous proteins. The E2 and E^{rns} glycoproteins of CSFV were expressed using recombinant NDV (rNDV). The rNDV expressing E2 and E^{rns} proteins showed effective CSFV neutralization antibody titer upon vaccination studies in pigs. The vaccinated serum samples showed neutralization of heterologous CSFV strains. A diagnostic based on the principle of indirect ELISA was developed using rNDV expressed E2 and Erns proteins of CSFV. The rescued rNDV containing E2 and E^{rns} proteins of CSFV were used to develop a diagnostic based on indirect ELISA. This proposed methodology gave us an insight that the E2 protein-based diagnostic is better as compared to E^{rns}. It was also observed that addition of E^{rns} along with E2 protein reduced the efficacy of the E2 based diagnostic. The proposed methodology could be an economical alternative to existing vaccine and diagnostic for CSFV control and detection, respectively, in pigs.

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Chapter I

Introduction and review of literature

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Chapter I

Introduction and review of literature

1.1 Introduction

Classical swine fever virus (CSFV) is a causative agent of an infectious disease in swine. The infection of CSFV affects domestic and wild pigs with varying degrees of pathogenicity. CSFV infection has been reported from Asia, parts of Africa, Central and South America and Europe (Artois, Delahay et al. 2001, Flores-Gutierrez and Infante 2008, Barman, Bora et al. 2016). More recently, countries like Madagascar, Singapore, Laos, Lithuania, Myanmar, Colombia, and the Republic of Korea also reported the CSFV outbreaks (Ji, Guo et al. 2015). The disease has been eradicated from Australia, North America, and New Zealand (OIE 2008). Persistent high fever, staggering gait, huddling, diarrhoea, cyanosis of the skin and haemorrhages are the major clinical symptoms of the CSFV infection (Ressang 1973, OIE 2008). CSFV infection to the pregnant sow could infect the embryo leading to mummification, abortion, the birth of weak piglets and death (OIE 2008). The piglets that survive the infection may act as a carrier of the disease. Prevention and vaccination is the only way to protect pigs from CSFV infection.

CSFV belongs to the family *Flaviviridae* in the genus *Pestivirus.* The member of this genus is structurally, genetically, and antigenically similar to bovine viral diarrhea virus (BVDV) and Border disease virus (BDV), which can infect ruminants and swine (Carbrey, Stewart et al. 1976). The virion of CSFV is enveloped and contains a positive strand RNA genome of approximately 12.3 kb size (Figure : 1.1) (Meyers, Rumenapf et al. 1989). The genome of CSFV encodes a single large open reading frame (ORF) flanked by untranslated regions (UTRs) (Vilcek, Leskova et al. 2014). The entire ORF encodes a polypeptide of approximately 3900 amino acids, which is processed to yield four structural (C, E^{rns}, E1, and E2) and eight nonstructural proteins (N^{pro}, P^{7}, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Figure: 1.2) (Lowings, Ibata et al. 1996, Meyers, Thiel et al. 1996). The structural and nonstructural proteins are processed by the cellular signalase and viral proteases, respectively (Elbers, Tautz et al. 1996, Lamp, Riedel et al. 2013)

The E2 is a major envelope glycoprotein that resides on the surface of CSFV and is crucial to induce host immune response during infection (Qi, Zhang et al. 2009). The E2 protein of CSFV contains four antigenic determinant regions, responsible for protective immunity against its infection (Greiser-Wilke, Moennig et al. 1990, Rumenapf, Stark et al. 1991, van Rijn, Bossers et al. 1996). In addition, E2 contains linear stretches of amino acid sequence motif, which serves as an epitopic marker for CSFV (Yu, Wang et al. 1996, Lin, Lin et al. 2000). The E2 protein has been explored to design DNA and subunit vaccines against CSFV infection (Bouma, de Smit et al. 1999, Beer, Reimann et al. 2007, Qi, Liu et al. 2008, Lin, Deng et al. 2012, Huang, Deng et al. 2014). Detection of E2 antibodies in the serum of CSFV infected animals is an easy and reliable way for its diagnosis. The E^{rns} is a second major immunogenic protein of CSFV that mediates its neutralization (Zhang, Yu et al. 2006). The E^{rns} protein is also involved in the entry of CSFV into the host cell (Konig, Lengsfeld et al. 1995, Hulst, van Gennip et al. 2000).

Virus neutralization test (VNT), fluorescent antibody test (FAT), immunoperoxidase staining, ELISA, virus isolation in cell culture and reverse transcription-polymerase chain reaction (RT-PCR) are some of the methods recommended by OIE to detect CSFV infection (De smit A.J 1994 , Colijn, Bloemraad et al. 1997, Hoffmann, Beer et al. 2005). However, ELISA has been proven sensitive and valuable tool to detect CSFV infection from the field samples. Previously, E2 protein-based ELISA has been developed for detection of CSFV specific antibodies in the serum samples (Lin, Zheng et al. 2010, Li, Mao et al. 2013, Cheng, Wu et al. 2014, Cheng, Pan et al. 2015, Kumar, Barman et al. 2016). Although bacterial expressed E2 based ELISA is available commercially, it is not glycosylated and folded in its native form. Moreover, the cost of its production is often high leading to a major constraint for the farmers in the developing nations. There is a need to produce a more effective, easy to produce and cost effective CSFV detection kit.

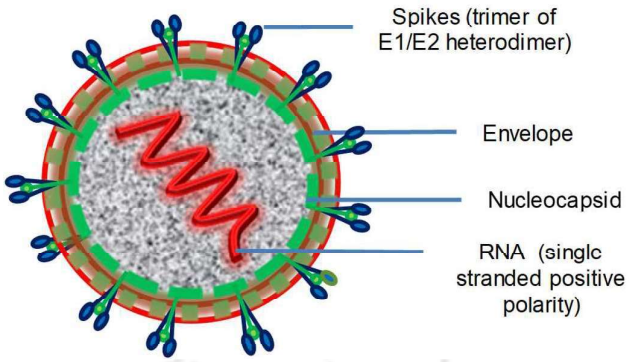


Figure: 1.1 Schematic representation of the CSFV virion.

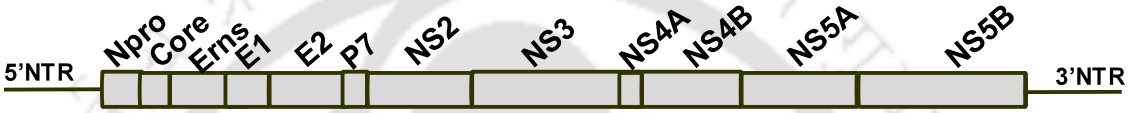


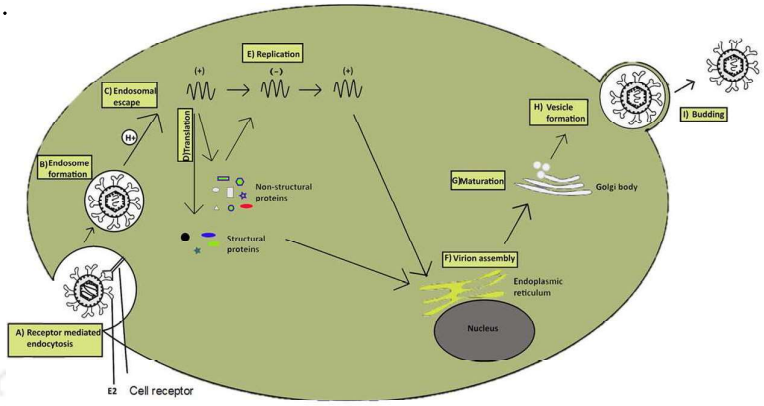
Figure: 1.2 Schematic representation of the CSFV genome.

1.1.1 Life cycle of CSFV

The lifecycle of CSFV has following essential steps: adsorption, entry, translation, protein maturation, genome replication (RNA replication), virus assembly, and the release. The infection steps are summarized in figure 1.3. The CSFV infection is initiated by the attachment of surface exposed virus proteins E^{rns}, E1, and E2 and the cellular receptors on the cell surface. Entry is accomplished by clathrin-dependent endocytosis as has been observed with other enveloped viruses that invade the host cell via receptor-mediated endocytosis. They require an acidic pH for fusion. Following un-coating of the nucleocapsid, the RNA genome is release into the cytoplasm. Repeated transcription and translation causes the release of virion particles.

Packaging of specific RNA genome inside the core or C protein depends on many factors like secondary structure of RNA and electrostatic characteristic of core protein. Even, repulsion by the negative charges on the phosphate groups of the nucleotide impede to cram into small protein-pocket; other counteracting ions, polyamines, and nucleic acid binding proteins package along with genome into protein pocket. The overall charge of specific RNA and core protein are responsible to eliminate the nonspecific packaging. Different viruses have their own specific signal sequence of nucleic acid, which recognize by core protein, viz psi sequence of murine retrovirus, other packaging signal, MS2 protein of picornavirus, alphavirus interact to hairpin of positive sense genome. N

protein of influenza has same role.(Rao 2006, Rulli, Hibbert et al. 2007, Brooke, Ince et al. 2014).Many research group illustrated the self-assembly of RNA and capsid in-vitro requires a Specific High Protein/RNA Mas Ratio. The nodaviral RNA2 requires N terminal of capsid protein.(Marshall and Schneemann 2001, Cadena-Nava, Comas-Garcia et al. 2012).



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1.1.2 Transmission

CSF is highly contagious and infected pigs act as carriers of CSFV. Blood, secretions, excretions, and tissues contain infectious CSFV particles. Shedding of virus begins before the onset of clinical signs, and occurs throughout the course of the disease. Transmission of the disease occurs by the oral or oronasal routes.

1.1.3 Morbidity and Mortality

The severity of the disease depends on the CSFV strains; while some strains cause acute conditions with high mortality rates, others can result in mild or even subclinical conditions. The morbidity and mortality rates are high during acute infections, and the fatality rate can approach 100%. Morbidity and mortality are lower in sub-acute cases. Chronic infections are always fatal but may affect only a few animals in a herd. The age

and immune status of the animals also affect the course of the disease, with lower mortality rates in adult pigs than younger animals.

1.1.4 Diagnosis

CSFV can be diagnosed by detecting the viral antigens or nucleic acids in the whole blood or tissue samples. Direct immunofluorescence techniques can be used to detect the CSFV antigens. The neutralization of virus through suspected serum is the gold standard to confirm the disease. The treatment of CSFV is not available and only vaccination can prevent the disease.

1.1.5 Newcastle disease virus as a vaccine vector

Viral vectors are very efficient to express different foreign proteins (Shimotohno and Temin 1981, Wei, Gibson et al. 1981, Tabin, Hoffmann et al. 1982, Rosenberg, Aebersold et al. 1990). Among all, Newcastle disease virus (NDV) is a choice of vector to express foreign proteins because of its unique properties (Figure: 1.4). NDV can be cultured with high titers in embryonated chicken eggs and in cell culture (McGinnes, Pantua et al. 2006, Arifin, Mel et al. 2010). In addition, the NDV infects through the intranasal route and induces a high antibody response (Ukai and Sakakura 1992, Takada and Kida 1996). Reverse genetics technique could lead us to develop recombinant NDV (rNDV) as a vector to express foreign genes of many animal and human proteins (Huang, Elankumaran et al. 2004, Park, Steel et al. 2006, DiNapoli, Kotelkin et al. 2007, Molouki and Peeters 2017).

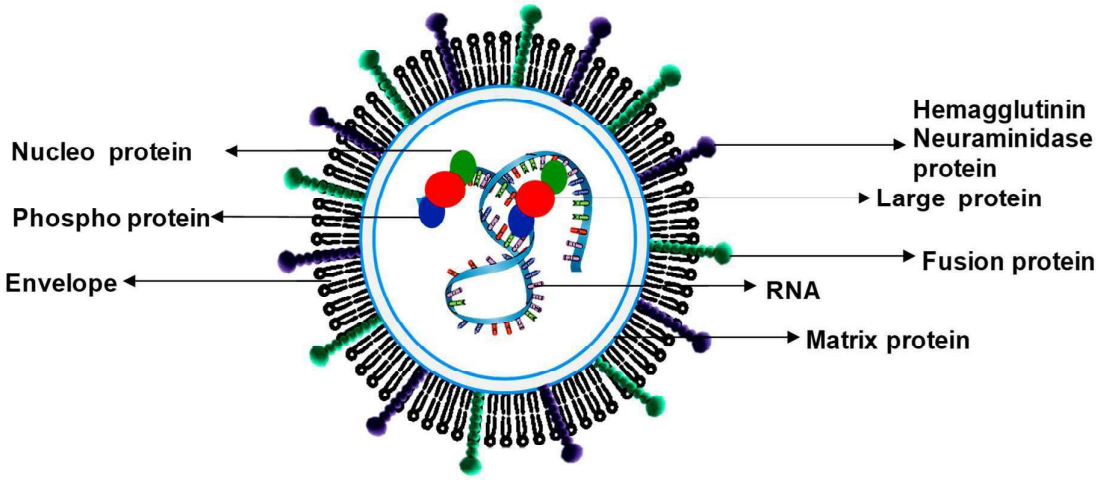

Figure: 1.4 Schematic representation of Newcastle disease virus

NDV has single stranded negative sense RNA genome and encodes for six different proteins in order of nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemeagglutinin-neuraminidase (HN) and large polymerase protein (L) from 3'-N-P-M-F-HN-L-5' (Figure: 1.5). The N, P, and L are polymerase complex protein while HN and F proteins are surface glycoproteins. Currently major attention of NDV pathogenicity has been focused towards the cleavage site of the F protein. Strains of NDV have monobasic or polybasic amino acids at the cleavage site where the cellular protease can act and cause its localized or systemic spread, respectively (Nagai, Klenk et al. 1976, Toyoda, Sakaguchi et al. 1987).

Figure: 1.5 Genome organization of Newcastle disease virus. Numerical value shows the number of nucleotides in each gene.

1.1.6 Recovery systems for recombinant NDV (rNDV)

The recovery of rNDV required a host-restricted recombinant vaccinia virus (MVA) and human cells such as human epidermoid carcinoma (HEp-2) for transfection (Figure: 1.5).

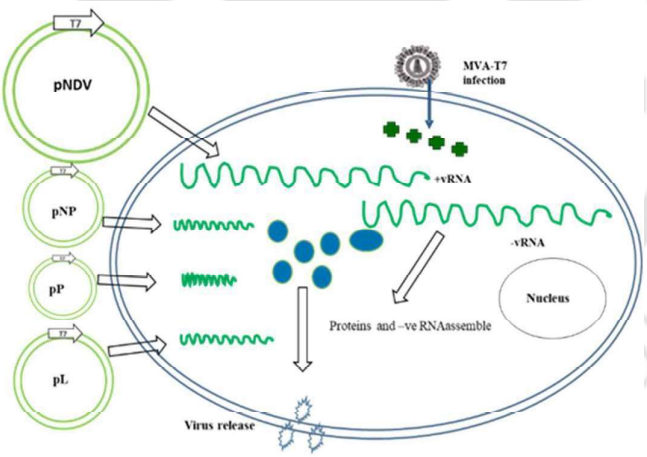

Figure: 1.6 The reverse genetics protocol employed for generating recombinant Newcastle disease viruses (NDV). The full-length antigenomic cDNA of NDV and the support plasmids encoding NP, P, and L proteins were transfected into human epidermoid carcinoma (HEp-2) cells that were pre-infected with recombinant vaccinia virus (MVA), to recover the recombinant virus. The recovered recombinant virus was then amplified in embryonated chicken eggs.

1.1.7 Rationale of the study

The modular nature of NDV genomes facilitates incorporating and engineering additional genes within its genome. The expression of foreign genes depends on the

addition of flanking transcription start and stop signals which are recognized by RdRp of the virus. However, levels of expression depend on the insertion site within the NDV genome due to gradient nature of its transcript. We believe that an rNDV vaccine will be highly effective in controlling CSFV infection in pigs. The rNDV vaccines can be administered by spray or in drinking water, which will allow vaccination of a large number of pigs in a short time. Moreover, the use of rNDV for immunization has the advantage that the foreign antigens are expressed and presented naturally, after infection.

There are some characteristics of NDV that makes it an ideal viral vector candidate.

1. NDV grows to very high titers not only in egg's embryo but also in many cell lines and it elicits strong humoral and cellular immune responses *in vivo*.
2. Naturally, NDV infects via respiratory and alimentary tract of mucosal surfaces, so it is especially useful to deliver protective antigens against respiratory disease.
3. rNDV would also have advantages over other live recombinant vaccine vectors as the foreign protein can be expressed with only a few NDV proteins.
4. NDV multiplies in the cytoplasm of the infected cells without having DNA intermediate phase, which finishes the problem of integration of virus 'genome into the host DNA. This virus does not undergo genetic recombination.
5. Generation of novel deletion mutants, a highly stable and efficacious vaccine, therefore, becomes a desired objective.
6. A recombinant chimeric NDV vaccine that allows serological differentiation between vaccinated and infected animals has been generated.
7. Expression of foreign proteins' level is quite high and the inserted genes are stable after many infections *in vitro* and *in vivo* (Nakaya, Cros et al. 2001).

The rNDV is an excellent vaccine vector for veterinary and human pathogens. rNDV expressing VP2 protein showed dual protection against NDV as well as infectious Bursal disease infection in chickens (Huang, Elankumaran et al. 2004). The HA protein of H5N1 and H7N7 influenza expressed in rNDV (Park, Steel et al. 2006) gives immunity against both influenza and NDV virus infection in chickens. rNDV containing glycoprotein of Rift Valley fever virus gN and gG has been shown to protect mice and lambs, against this virus infection through eliciting antibody response (Kortekaas, de Boer et al. 2010). Nipah virus G and F proteins expressed by rNDV causes production of neutralizing antibody in mice and pigs (Kong, Wen et al. 2012).

1.2 Objectives

1. Development of Indirect ELISA using CSFV surface protein expressed in bacteria.
   (a) Cloning of immunogenic protein
   (b) Expression and purification
2. Evaluation of recombinant CSFV proteins expressed using NDV vector
   (a) Construction of recombinant NDV and recovering of the virus.
   (b) Cloning and expression of the foreign epitopic protein of CSFV in rNDV.
3. Evaluation of vaccine efficacy of rNDV expressing immunogenic proteins of CSFV
   (a) Vaccination study in pigs
   (b) Neutralization of CSFV
4. Evaluation of rNDV expressing CSFV proteins as a diagnostics
   (a) Formulation of an indirect ELISA

1.3 Review of Literature

1.3.1 History and global scenario

Classical swine fever (CSF) has been recognized as a major pig pathogen and is a notified condition by the world organization for animal health. The etiology of the disease was identified as a virus that could pass through the filter (Edwards, Fukusho et al. 2000). The abbreviation HCV had been used for many years for the hog cholera virus, but changed to classical swine fever virus (CSFV) because of its redundancy with the hepatitis C virus. The disease is endemic in Asia, Central and South America, and parts of Europe and Africa (OIE 2008). CSFV has been eradicated from the USA and the UK but an outbreak has been reported in East Anglia in 2000 (Paton 2008). It is believed that CSFV has been eradicated from countries like Australia, Canada, Ireland, New Zealand, and Scandinavia owing to the absence of any outbreak since the last two decades.

CSF has not been reported from the southern states of Brazil, including Rio Grande do sul, Santa Catarina, and Parana since 1988 save an isolated incident in 1997 due to a strict vaccination procedure using C-strain of CSFV (Edwards, Fukusho et al. 2000).

CSFV was introduced into Cuba in 1930 from the USA and was kept under control until 1993 through regimes of vaccination. Uruguay is free of CSF according to records since 1991. The first report of CSF in Haiti is documented to have taken place in August 1996 in Port-au-Prince. However, the origin of the disease, in this case, was not

known. CSF was reported in the Dominican Republic in June 1997. Without vaccination, Chile has declared itself to be free of CSF since April 1998 (Edwards, Fukusho et al. 2000).

In the European Union (EU), major epidemics of CSFV have been recorded in the last decade. The first report was in Belgium in 1988. Ignorance of vaccination policy in the EU (1990) caused several disastrous CSF outbreaks in the 1990s (Bartak and Greiser-Wilke 2000, Fritzemeier, Teuffert et al. 2000). In Germany (1990-1998), 424 outbreaks of CSF have been reported in domestic pigs as well as in wild boar until 2009. Hence, to eradicate the disease, vaccination of wild boar was performed until March 2012 (Postel, Moennig et al. 2013). Several outbreaks of CSF were recorded in Croatia from 1997-2009 (Jemersic, Greiser-Wilke et al. 2003). CSF virus was reported in the Netherlands in early 1997 (Elber, Stegeman et al. 1999). The virus was then further spread from The Netherlands to Italy, Spain and Belgium in 1997 (Stadejek, Vilcek et al. 1997, Bartak and Greiser-Wilke 2000, Dewulf, Laevens et al. 2000, Greiser-Wilke, Fritzemeier et al. 2000). CSFV virus was introduced into the UK in 2000 (Stegeman, Elbers et al. 2000). In Switzerland, CSF was detected in May 1998 in Canton Ticino (Leifer, Depner et al. 2009) and in Spain between 2001 and 2002 which affected 49 herds (Allepuz, Casal et al. 2007). In the regions of the French-German border, CSF was present in wild boar over a longer period. Last attacks to be documented in Germany were in 2009 in the federal states of Rhineland-Palatinate and North Rhine-Westphalia (OIE, 2012). Persistence of CSF at low levels in the wild boar population, despite vaccination was found to hamper successful eradication of the disease (Leifer, Everett et al. , Leifer, Hoffmann et al.). Amongst the newly joined member states of the EU as of 2004, only Slovakia continued to be riddled with CSF in domestic pigs.

After a complete absence of the disease for 62 years, CSF re-emerged in Northern Israel in domestic pigs and wild boar, in February 2009. On the basis of phylogenetic analysis, the strain that affected the herds were found to be genetically most similar to a Chinese CSF virus strain (David, Edri et al. 2011).

CSF was first reported in Japan in 1888. No reports were documented between 1975 and 1978 (Edwards, Fukusho et al. 2000). In the rest of South-East Asia, outbreaks of CSF occur regularly in most countries except for Japan. Between 1996 and 1997, the number of cases of CSF was nearly zero in the Singapore and Thailand. Korea and Myanmar also brag a low incidence of the disease during the above time period. However, Vietnam and Indonesia have high numbers of incidence with Indonesia having

the highest number of reports of CSF between 1996 and 1997(Ji, Guo et al. 2015). The first suspected case of CSF in Indonesia was reported in Sumba Timur and Flores Timur districts in 1997. In 2000, cases were reported in Sikka district. In July 2002, the first case of CSFV was detected on Alor Island (Sawford, Geong et al. 2015). CSF is considered to be endemic in Mongolia with outbreaks in 2007, 2008, 2011, 2012, 2014, and 2015. Based on phylogenetic analysis, they were found to be closely related to isolates from China and Korea (Enkhbold, Shatar et al. 2017).

In china, 285 CSF outbreaks were reported in 12 provinces and autonomous regions by the Veterinary Bureau of Ministry of Agriculture (MOA) 2013. To date, CSF has not been brought completely under control owing to its sporadic and endemic nature in many areas of China (Tu, Lu et al. 2001, Zhang, Leng et al. 2015).

Two CSF outbreaks took place in the Republic of Korea during 1988 and 2003. Phylogenetic analysis results attribute the outbreaks to the transmission of a new strain or strains from the neighbouring countries of China and Taiwan (Cha, Choi et al. 2007). In 1999, Jeju island of South Korea was declared free of CSF. CSFV isolates from Nepal showed higher genetic similarity to those from India. Both Nepal and India were therefore considered as one epidemiological unit (Postel, Jha et al. 2013).

The first documented report of CSF in New South Wales, Australia was in 1902. During the period of 1928-1942, Australia remained free of CSF (Edgar, 1946). Countries including Bhutan, Cambodia, Philippines, and Vietnam reported CSF outbreaks in both 2011-12 and 2013-14 (Ji, Guo et al. 2015).

A phylogenetic analysis of 106 viruses isolated from 20 countries over a period of 52 years (1945-1997) showed that the viruses could be categorised into two main groups. Asian and South American isolates from the 1980s along with old European and American isolates were categorised into group 1 while recent European viruses from the 1980s and 1990s belonged to group 2. Five CSFV isolates from Germany, Netherlands, and Italy isolated in the year of 1997 clustered together indicating a common origin for these outbreaks (Bjorklund, Lowings et al. 1999).

1.3.1.1 Scenario in India

Different parts of India reported the outbreaks of CSFV (Bhaskar, Ravishankar et al. 2015, Choori, Patil et al. 2015). Although the disease has been reported from most of the states in India, North East part of India due to the higher density of the pig population frequently reports its outbreak. The North East part of India shares a large porous

boundary with China, Bangladesh, Bhutan, and Myanmar. Therefore, this region is prone to transboundary transmission of exotic CSFV strains (Barman, Gupt et al. 2010, Sarma, Mishra et al. 2011, Khatoon, Barman et al. 2017). In addition, pig rearing is an important agricultural activity in the North East states of India and has an important role in the socioeconomic development of the states. The prevalence of CSFV has been reported in wild hog and piglets from different parts of Assam (Barman, Bora et al. 2014, Khatoon, Barman et al. 2017). CSFV isolates from the field outbreaks from various regions of India during 2006-2009 were subjected to phylogenetic analysis. The results indicated plausible Chinese origin and provided evidence of CSFV movement routes within the South East Asia region (Patil, Hemadri et al. 2012). The nucleotide sequence analysis of different strains of CSFV from India suggested the circulation of subgenogroup 1.1 (Choori, Patil et al. 2015). Occasionally, subgenogroup 2 has also been reported from India (Desai, Sharma et al. 2010, Chakraborty, Chakraborty et al. 2011).

1.3.2 Probable reasons for the persistence of CSF over the years and possible solutions

While faulty regulations have led to the production of inefficient vaccines, the failure to follow immunisation schedules accurately has led to disease outcome. Unrestrained transport of diseased animals by illegal traders has resulted in the far-ranging spread of the disease as a part of international trade. A legal setup that lays down the framework for CSF control is absent which has further aggravated the situation. Successful eradication of virulent virus strains may have resulted in the evolution of less virulent CSFV which might also be another reason for the re-emergence of CSF. Asymptomatic progression of the disease may result in delayed diagnosis and therefore the untimely implementation of control measures, thereby causing further spread of the disease (Edwards, Fukusho et al. 2000, Blome, Staubach et al. 2017).

Several measures, including effective marker vaccine development, regional and national eradication campaigns, and vaccination drives coupled with serosurveillance can be taken up to propel the complete control and eradication of CSF worldwide. Multiplex assays which can successfully detect diverse pestiviruses (both existing and emerging) can keep a check on viral co-infections. A deeper knowledge of the replication and infection cycle of the viruses and its interaction patterns with the host can help develop effective strategies for CSF control. Identification of host and viral interacting partners at

the time of infection can help provide powerful insights into the virus life cycle (Li, Li et al. 2013, He, Ling et al. 2014, Lin, Wang et al. 2017).

1.3.3 Classical swine fever virus

The family *Flaviviridae* has four genus *hepacivirus*, *flavivirus*, *pestivirus*, and *pegivirus.* CSFV resides in the genus *pestivirus* and is antigenically and structurally similar to other species of virus-like bovine viral diarrhoea viruses (BVDV-1 and -2) and Border disease virus (BDV-2) (OIE 2008, Postel, Schmeiser et al. 2015). The incubation period of CSFV varies from 2 to 15 days. In acute conditions, it is often 3-7 days. In field conditions, between 2 to 4 weeks or a longer time can be taken for the infection to appear in a herd (CFSPH 2015). The CSFV is an icosahedral enveloped virus with a diameter of 40-60 nm (Horzinek 1967, Moennig 1992). It contains a positive sense RNA of approximately 12.3 kb size, which translates to a single large open reading frame (ORF) (Meyers, Rumenapf et al. 1989, Moormann 1996). The genome of CSFV is flanked by untranslated regions (UTRs) flanking the entire ORF that encode a polypeptide of approximately 3900 amino acids (Rümenapf, Meyers et al. 1991, Collett 1992). The polyprotein gives four structural C, E^{rns}, E1 and E2 and eight nonstructural proteins (Npro, P70, NS2, NS3, NS4A, NS4B, NS5A, NS5B) after processing by the cellular and viral proteases (Rümenapf, Meyers et al. 1991, Thiel, Stark et al. 1991, Rumenapf, Unger et al. 1993, Tautz, Elbers et al. 1997, Bintintan and Meyers 2010). The genomic RNA lacks a 5′ cap-structure and a 3′ poly (A) tail but comprises structured 5′UTR and 3′UTR regions. The internal ribosome entry site (IRES), present at the 5′UTR helps in cap-independent translation of polyprotein (Jang, Krausslich et al. 1988). CSFV has three phylogenetic groups (1, 2, and 3), based on E2 sequence. Each group is subdivided into different sub-genotypes like (1.1 to 1.3; 2.1 to 2.3; and 3.1 to 3.4) (Paton D. J. and Bjorklund H. 2000, Greiser-Wilke I. 2006, Postel, Schmeiser et al. 2012). The CSFV genotype 1 and 2 are mostly circulating in China and Europe, while genotype 3 strains are mostly isolated from Asia (Paton D. J. and Bjorklund H. 2000, Sandvik T. 2000, Stegeman A. 2000, Leifer I. and . 2010, Graham S. P. and R. 2012).

1.3.4 Viral proteins

CSFV has four structural C, E^{rns}, E1, and E2 and eight non-structural proteins (Npro, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B).

Core (C) protein

The core (C) protein is a small basic protein of 14.3 kDa size with 99 amino acids (Meyers, Rumenapf et al. 1989, Meyers, Thiel et al. 1996, Zhang H. 2011, Ji, Guo et al. 2015). It protects RNA after making RNA–protein complex (Thiel, Stark et al. 1991, Meyers, Thiel et al. 1996). It is released from polyprotein by Npro and host signal peptidase (Heimann, Roman-Sosa et al. 2006, Gottipati, Ruggli et al. 2013). The amino acids at positions 12, 26–30, and 40–44 of the C protein play a vital role in the production of virus progeny (Riedel, Lamp et al. 2010). The C protein is also involved in the SUMOylation pathway, which helps in the CSFV replication and its escape from the host immune response (Gladue, Holinka et al. 2010). The C protein is also an antagonist of the host interferon response (Riedel, Lamp et al. 2017).

E2 protein

The E2 (50- 55kDa, 373 aa) protein is an immunogenic surface glycoprotein. It has been used for vaccine and diagnostics against CSFV (Beer, Reimann et al. 2007, Reimann, Depner et al. 2010, Li, Wang et al. 2012, Kumar, Barman et al. 2016). It has also been used as a DNA vaccine (Li, Mao et al. 2015). The full length of E2 protein gene nucleotide sequence is being used for the genotype classification of CSFV strains (Postel, Schmeiser et al. 2012). The E2 has four immunogenic domains (A to D) at its N-terminal. The structural study suggests E2 role in cell entry (El Omari, Iourin et al. 2013). A linear epitope of nine amino acids ^{829}TAVSPTTLR^{837} located in the A domain, is highly conserved and specific to CSFV. This linear epitope is involved in virulence and evasion from the host immune response (Risatti, Holinka et al. 2006, Leifer, Blome et al. 2012). The E2 is a type I transmembrane protein and its C-terminal anchors to the viral envelope (Risatti, Holinka et al. 2007, Li, Wang et al. 2013). It elicits neutralizing antibody production after the infection in the host (Qi, Liu et al. 2008, Qi, Zhang et al. 2009). E2 forms a homodimer with itself and heterodimer with E1. A different kind of non-structural fusion protein E2-P7 (443 amino acid) has been reported, which is formed due to inefficient cleavage between E2 and P7 protein (Elbers, Tautz et al. 1996).

E^{rns} protein

The E^{rns} protein (41-44 kDa, 227 aa) protrudes on the surface of the CSFV virion (van Gennip, van Rijn et al. 2000). The E^{rns} is considered to be the second most immunogenic protein after E2 and is able to elicit a neutralizing antibody response

(Weiland, Ahl et al. 1992, Maurer, Stettler et al. 2005, Zhang, Yu et al. 2006). It has been reported that E^{rns} assists in CSFV entry into the host cell (Hulst, van Gennip et al. 2000). The N-linked glycans impart more than 50% of the molecular mass for E^{rns} (Rumenapf, Unger et al. 1993). The E^{rns} exists as a homodimer in which cysteine disulfide bond at a position 171 has a crucial role. The CSFV strains with the absence of Cys^{171} show non-cap availability in dimerization and virus attenuation, but replication competence (Tews, Schurmann et al. 2009). The E^{rns} attach weakly to the virion due to the absence of membrane-anchored domain. It comprises an amphipathic helix anchor, which assists to its membrane attachment (Fetzer, Tews et al. 2005, Tews and Meyers 2007). The ribonuclease (RNase) activity is a unique feature of the E^{rns} protein (Kumar, Kumar et al. 2018). The E^{rns} shows homology with T2 RNase superfamily (Schneider, Unger et al. 1993) and has secretory nature and circulates in body fluids of the infected host (Rumenapf, Unger et al. 1993, Magkouras, Matzener et al. 2008). It regulates RNA synthesis in infected cells and inhibits dsRNA induced IFN-β production during its replication (Xia, Chen et al. 2007). Its attachment to the cell surface is not only restricted to swine cells but can attach to other unnatural cells (Hulst and Moormann 1997). In addition, it is reported to cause apoptosis in lymphocytes and suppress early-stage immunization of the infected animal (Bruschke, Hulst et al. 1997).

E1 protein

E1 protein is also (33 kDa, 195 aa) an envelope glycoprotein. The type I transmembrane E1 protein co-attach to host receptors, and exists as E1-E2 dimer on the virus envelope (Weiland, Stark et al. 1990). The E1 and E2 heterodimers facilitate the CSFV attachment and invasion to the host immunity (Wang, Nie et al. 2004, El Omari, Iourin et al. 2013). E1 has three N-linked glycosylation sites at positions 500, 513 and 594, which play an important role in virus viability. The modification in glycosylation could lead to non-viable CSFV and removal of any glycosylation sites caused attenuation of CSFV strains (Fernandez-Sainz, Holinka et al. 2009). These three glycosylation sites are highly conserved in CSFV whereas 513 and 594 glycosylation sites are conserved in other *pestiviruses* (Fernandez-Sainz, Holinka et al. 2009). The attenuation of CSFV strains is possible after modification of E1 glycosylation patterns (Ganges, Nunez et al. 2008). The C-terminal region acts as a membrane-spanning domain to anchor in the envelope of the virus (Thiel, Stark et al. 1991).

1.3.5 Replication cycle of CSFV

Adsorption and entry of the CSFV are mediated through viral surface proteins E^{rns}, E1, and E2. Different types of cell receptors such as heparan sulfate (HS) (Hulst, van Gennip et al. 2000, Hulst, van Gennip et al. 2001), Laminin (Chen, He et al. 2015) low-density-lipoprotein (LDL) receptor (Agnello, Abel et al. 1999) and CD46 receptor (Maurer, Krey et al. 2004) have been suggested for the CSFV entry into the host cells. The interaction of cellular receptors and glycoproteins E2 homodimers or heterodimers of E2-E1 (Weiland, Stark et al. 1990, Thiel, Stark et al. 1991) and E2- E^{rns} (Lazar, Zitzmann et al. 2003) are involved in CSFV entry. It has been shown that E^{rns} could interact with glycosaminoglycans of the host cell (Iqbal, Flick-Smith et al. 2000). Entry of the virus is mediated by clathrin-dependent endocytosis as reported for other flaviviruses (Chu and Ng 2004, Lecot, Belouzard et al. 2005). The virion is engulfed at clathrin-coated pits and vesicle promotes early endosomal pathway. Further, the low pH triggers conformational changes of the viral glycoproteins, which cause its fusion with the endosomal membranes and the RNA genome is released into the cytoplasm (Lindenbach and Rice 1999, Krey, Thiel et al. 2005). Ribosome and IRES interaction initiates cap-independent translation of the viral polyproteins (Poole, Wang et al. 1995, Pestova and Hellen 1999). The processing of polyprotein into different precursor proteins takes place through viral auto-protease or cellular signalase (Rumenapf, Unger et al. 1993). In the beginning, Npro is auto-cleaved from the polyprotein (Stark, Meyers et al. 1993) followed by the cleavage of structural proteins, p7 and NS2-3 by the cellular proteases. The non-structural proteins are processed primarily in the cytoplasm while the structural proteins are also reported to be processed in the ER lumen (Weihofen, Binns et al. 2002).

Viral RNA replication starts through the synthesis of a negative-complementary strand to genomic RNA with the help of cis-acting element of RNA genome. The negative strand acts as the template for synthesis of positive-strand RNA (Behrens, Grassmann et al. 1998). The NS5B protein facilitates the incorporation of a priming GTP that acts as the primer for replication (Choi, Groarke et al. 2004). A highly conserved 5´-GUAU sequence interacts with a 3´UTR-AUAC sequence, which helps in the cyclization of the viral genome. Any mutation in these sequences is reported to impair the CSFV replication (Frolov, McBride et al. 1998, Becher, Orlich et al. 2000). At any given time, positive-strand RNAs are present in excess over the negative strand RNA (Mittelholzer, Moser et al. 2000).

The knowledge of CSFV assembly is not well understood. Assembly is presumed to occur in the ER lumen by encapsulating the RNA genome by C-proteins (Kunkel and Watowich 2002, Murray, Marcotrigiano et al. 2008). Surface glycoproteins accumulate inside the ER membrane, and nucleocapsid is enclosed by it in the ER lumen (Murray, Jones et al. 2008). The mature virus is released by exocytosis and p7 protein destabilizes the membrane prior to exocytosis and functions as a viroporin (Harada, Tautz et al. 2000, Chandler, Penin et al. 2012).

1.3.6 Diagnostic development through PCR and its different variants

At present, the detection of CSFV genome through RT-PCR is a good and reliable way to identify the virus from the tissue samples. Many diagnostic RT-PCR protocols have been developed targeting the 5'UTR, NS5A, and E2 segment of CSFV genome (Wirz, Tratschin et al. 1993, Hofmann, Brechtbuhl et al. 1994, Canal, Hotzel et al. 1996, Leifer, Blome et al. 2011, Postel, Schmeiser et al. 2012, Jiang, Gong et al. 2013). In addition, multiplex RT-PCR, real time PCR, reverse transcription loop-mediated isothermal amplification (RT-LAMP) and multiplex/ hot start multiplex PCR have also been reported to diagnose CSFV from field samples. The viral RNA from aerosol has been used to quantify different strains of CSFV following laboratory infection (Weesendorp, Stegeman et al. 2009). Furthermore, oral fluid from infected pigs was analyzed to diagnose CSFV nucleic acid (Petrini, Pierini et al. 2017).

Several real-time PCRs with SYBR green and TaqMan probing have been reported to diagnose CSFV (Risatti, Holinka et al. 2005, Ophuis, Morrissy et al. 2006, Lung, Pasick et al. 2016). They were later found to be more appropriate for pestivirus quantification (Jamnikar Ciglenecki, Grom et al. 2008). A high sensitive real-time PCR based assay with primer-probe energy transfer (PriProET) technology was claimed to detect 20 copies of cDNA (Liu, Xia et al. 2009, Zhang, Xia et al. 2010). A FAM-labeled TaqMan-probing at 5'UTR was claimed to show the sensitivity of approximately eight copies of CSFV genome (Hoffmann, Beer et al. 2005). The skin punch biopsies and nictitating membrane are suitable candidates for postmortem tissue in diagnosing of CSFV infection through any PCR based approach (Teifke, Lange et al. 2005, Kaden, Lange et al. 2007). It has been shown that the EvaGreen-based multiplex real-time PCR could diagnose multiple pig pathogens simultaneously (Rao, Wu et al. 2014, Shi, Liu et al. 2016).

1.3.7 Diagnostic through ELISA methods and its different variants

Different kinds of ELISA are being used to diagnose CSFV antibody using its proteins (Muller, Depner et al. 1996, Clavijo, Zhou et al. 1998, Clavijo, Lin et al. 2001, Clavijo, Lin et al. 2001, Langedijk, Middel et al. 2001, Huang, Chien et al. 2006, Sung, Kang et al. 2011, Wu, Chien et al. 2011, Yang, Li et al. 2012, Aebischer, Muller et al. 2013, Li, Mao et al. 2013, Cheng, Wu et al. 2014, Luo, Li et al. 2015, Pannhorst, Frohlich et al. 2015, Kumar, Barman et al. 2016, Ji, Luo et al. 2018) The complex-trapping-blocking (CTB) two step ELISA (Wensvoort, Bloemraad et al. 1988) and single step ELISA using recombinant E2 protein have been used to detect CSFV antibodies (Colijn, Bloemraad et al. 1997). Indirect ELISA and Avidity-Blocking ELISA based on single serum dilution method were also developed through recombinant protein for pestivirus (Franco Mahecha, Ogas Castells et al. 2011, Kumar, Barman et al. 2016),). A bacterial expressed recombinant quadruple epitopic peptide of E2 has been reported to develop an indirect ELISA (Lin, Zheng et al. 2010). The tandem repeat of TAVSPTTLR antigen as tetramer or hexamer has been reported as a good candidate for serological related studies of CSFV (Qi, Zhang et al. 2009). An immune-chromatographic strip was developed using epitopic gold labeled chimeric recombinant protein of E^{rns} and E2 for rapid detection of antibodies against CSFV (Li, Wang et al. 2012). The kits such as Chekit-ELISA and Herd-Chek-ELISA (IDEXX) are available commercially (Hergarten, Hurter et al. 2001). The study also showed that the Chekit* CSF-marker, an E^{rns} ELISA did not differentiate between antibodies against ruminant pestiviruses and CSFV, however, this could be achieved by PrioCHECK CSFV E^{rns} ELISA (Schroeder, von Rosen et al. 2012, Pannhorst, Frohlich et al. 2015). The E2 fused with immunoglobulin kappa signal peptide in the lentivirus vector is used to generate a cell-secreting E2 through human embryonic kidney cell line and used to coat for ELISA (Cheng, Pan et al. 2015).

The presence of antibody in the fecal sample showed high sensitivity in ELISA and also had CSFV neutralization ability (Seo, Sunwoo et al. 2012). Another immuno-chromatographic lateral flow assay was also developed through monoclonal antibody of CSFV(Sambandam, Angamuthu et al. 2017). A magneto-elastic (ME) sensor has been developed for CSFV detection through immobilized IgG based ME sensor, the resonance frequency of ME sensor increases with augmentation of CSFV concentration and confirms its presence (Guo, Gao et al. 2016).

1.3.8 Vaccines

According to the OIE, the CSFV vaccine should give protection within a period of two-weeks and immunity should last for six months. A single dose should be able to give quick and reliable protection. In addition, vaccination to pregnant sows does not cause trans-placental infection and congenital infection to the litters.

Different kinds of vaccines have been used for CSFV at laboratory and field level. Some are registered and some are being used in particular countries under the supervision of their government policies. The vaccines of CSFV include killed, oral, lapinized live attenuated, subunit, fusion protein based, chimeric, DNA, trans-complemented replicons, and viral vector based. These vaccines have their own advantages and disadvantages. Different reviews have been published highlighting the efficacy and developmental steps of CSFV vaccines (de Smit 2000, Beer, Reimann et al. 2007, Dong and Chen 2007, Blome, Staubach et al. 2017).

Live attenuated vaccines and its variants

Serial passage of a virulent strain in a non-natural or non-susceptible host could produce attenuated virus, which induces a strong protective immune response without a disease condition. The live attenuated Chinese "C strain" is the gold-standard vaccine against CSFV in Europe. The C-strain vaccine developed from serial passage of virulent Shimen strain in rabbits showed effective and rapid protection against CSFV (van Oirschot 2003, Beer, Reimann et al. 2007, Graham S. P. and R. 2012). The C strain CSFV showed clearance from the body secretion and excretion following 10-12 days post vaccination (Kaden, Lange et al. 2004). Phylogenetic analysis evidence raised confliction about the origin of C-strain from Shimen strain (Xia, Wahlberg et al. 2011). This might have happened due to probable mutation during passages or adaptation of Shimen strain in rabbits. A Mutation in the E2 protein gene after passaging or adaptation of lapinized vaccine strain in natural host cell line PK15 has been documented (Kumar, Barman et al. 2015). Several variations of C-strain are being used for vaccination, such as LPC, LC, RIEMSER, and RIEMS. The insertion or replacement of 12 nucleotides at 3'UTR led to attenuation of virulent CSFV and protection of the host against lethal challenge (Wang, Wang et al. 2008). The full genome sequencing of virulent and avirulent strains of CSFV gave us an insight into the location of attenuation features (Gupta, Saini et al. 2011, Tomar, Gupta et al. 2015).

Some live attenuated or chimeric vaccines are developed as a bait format for oral vaccination in wild boar and domestic pigs (Kaden, Lange et al. 2000, Kaden, Heyne et al. 2002, Koenig, Lange et al. 2007, Kaden, Lange et al. 2010, Dietze, Milicevic et al. 2013, Milicevic, Dietze et al. 2013, Renson, Le Dimna et al. 2013, Monger, Stegeman et al. 2016). It has been shown that the contaminated feed with Lom strain of CSFV caused induction of antibody against the vaccine virus (Kim, Song et al. 2008). The killed vaccine generally lacks the cell-mediated immune response and only induces a humoral response.

Live attenuated vaccines as a marker

Different live attenuated vaccines are reported as marker vaccine for CSFV with varied efficacy (Widjojoatmodjo, van Gennip et al. 2000, Frey, Bauhofer et al. 2006). The chimeric CSFV comprising envelope protein E^{rns} or E2 (antigenic part) of bovine viral diarrhoea virus has been studied as live attenuated marker vaccine (van Gennip, van Rijn et al. 2000).

Studies were also conducted to show the kinetics of maternally derived antibodies after intramuscular vaccination of live attenuated Suvaxyn(R) CSF marker vaccine (Schroder, Drager et al. 2016). Efficacy of Suvaxyn CSFV vaccine studied in the presence of BVDV antibodies showed that the preexisting antibody did not affect its efficacy (Drager, Schroder et al. 2016).

Marker vaccine based on recombinant protein

A marker vaccine based on peptide or whole protein is being used to differentiate infected from vaccinated animal (Dong, Qi et al. 2006). Several ways were tried to express the E2 protein as a vaccine candidate in pigs (Bouma, de Smit et al. 1999, Ahrens, Kaden et al. 2000, Bouma, De Smit et al. 2000, Lipowski, Drexler et al. 2000, Lin, Liu et al. 2009). The multi-peptide overlapping vaccine or peptide of E2 also showed protective immunity in rabbit and pig or elicit neutralizing antibody (Dong, Wei et al. 2002, Dong, Chen et al. 2005, Dong and Chen 2006, Dong, Qi et al. 2006, Tarradas, Monso et al. 2011, Li, Zhou et al. 2012). The epitope-based vaccine is able to induce protective immunity against CSF (Dong and Chen 2006). The intradermal and intramuscular administration of E2 subunit vaccine reduced the horizontal transmission of CSFV (Dortmans, Loeffen et al. 2008). The recombinant E2 loaded with PLGA microspheres showed high potential for its use as a mucosal route subunit vaccine

(Brandhonneur, Loizel et al. 2009). The yeast, baculovirus and mammalian expressed E2 proteins are also able to provide protection against CSF in pig (Lin, Liu et al. 2009, Lin, Deng et al. 2012, Hua, Huo et al. 2014) and rabbit (Zhang, Li et al. 2014). A recent study showed protection of E2 immunized pig against the heterologous strains of CSFV (Madera, Gong et al. 2016). The fused E2 and CD154 protein-based vaccine showed effective humoral and cellular immunity (Munoz-Gonzalez, Sordo et al. 2017, Suarez, Sordo et al. 2017, Munoz-Gonzalez, Sordo et al. 2018, Sordo, Suarez et al. 2018).

In contrast, Porcilis® Pesti, MSD Animal Health, Unterschleißheim, Germany (E2 subunit marker vaccine) is commercially available. Although this vaccine has been shown to be safe and protective with the limit spread of CSF(van Oirschot 1999, Ahrens, Kaden et al. 2000, Dewulf, Laevens et al. 2000, Lipowski, Drexler et al. 2000, de Smit, Bouma et al. 2001), it has drawbacks in terms of protection against trans-placental transmission and does not give early protection against CSF(Depner, Bouma et al. 2001, van Oirschot 2003, van Oirschot 2003). Therefore several research groups have looked for developing a next-generation marker vaccine that would be able to fulfill all demands like safety, efficacy, and DIVA potential, and market appeal (Beer, Reimann et al. 2007). To fulfill these demands many ideas have been evaluated or have evolved for different viral-based vectors like adenovirus (Hammond, Jansen et al. 2001, Li, Gao et al. 2016) vaccinia virus, pseudorabies virus(Lei, Xia et al. 2016) and Newcastle disease virus (our unpublished work). The adenoviral expressed E2, flanked with tissue plasminogen signal was infused with mammary gland of goat. The secreted E2 protein in milk provided long-lasting and early protection in pig (Toledo, Sanchez et al. 2008, Barrera, Sanchez et al. 2009, Barrera, Sanchez et al. 2010, Toledo, Barrera et al. 2010, Sanchez, Barrera et al. 2014).

DNA vaccine and its variance

The DNA vaccine based on E2 gene along with IL-18 and CD154 showed effective antibody titer against CSFV challenge (Wienhold, Armengol et al. 2005). The co-delivery of chemokine CCL20 in DNA vaccine of E2 showed an immunomodulatory effect in pigs (Tarradas, Alvarez et al. 2011). The chimeric DNA vaccine of Semliki forest virus replicon, NS-1–4 and E2 showed more efficient gene delivery (Sun, Li et al. 2011). Several groups have reported E2 based DNA vaccine with various degree of efficacy (Zhou, Lu et al. 2000, Ganges, Barrera et al. 2005, Sun, Zhao et al. 2008, Zhao, Sun et al. 2009, Tarradas, Argilaguet et al. 2010, Wan, Yi et al. 2010, Tian, Sun et al.

2012). The swine CD81 with E2 enhances cellular and humoral response in DNA vaccine (Li, Mao et al. 2015). Interestingly, the E2 with 5' signal sequence could give protection against CSFV in rabbit and pigs (Yu, Tu et al. 2001). The combined DNA vaccination strategy against antigens of foot-and-mouth disease virus (FMDV), pseudorabies virus and CSFV, caused a reduction in immunogenicity of FMDV antigen in mice (Wang, Guo et al. 2007). A group showed that the use of E2 DNA vaccine against CSFV is completely safe for both pig and environment (Cheng, Wang et al. 2005). The alphavirus-based DNA vaccine of Semliki Forest virus (SFV) containing E2 gene showed protection after administration in rabbits and pigs against CSF. They evaluated that SFV replicon-derived DNA vaccine could be a marker vaccine (Li, Qiu et al. 2007, Li, Zhao et al. 2007, Li, Zhao et al. 2007, Xia, Lei et al. 2016, Xia, Xiang et al. 2016). The *Salmonella choleraesuis* containing E2 gene of CSFV was used for oral and intramuscular vaccination in rabbit and mice (Qiao, Sun et al. 2005).

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Chapter II

Molecular and physical characterization of lapinized CSFV vaccine

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Chapter II

Molecular and physical characterization of lapinized CSFV vaccine

2.1 Abstract

The disease is endemic in many parts of the world and vaccination is the only way to protect the animals from CSFV infection. The lapinized vaccine strains are occasionally not protective because of animal to animal passage, inadequate vaccination strategy, suboptimal vaccine dose, and emergence of new variants. The surface glycoprotein E2 of CSFV is a major antigenic determinant and can modulate the disease outcome in porcine. In the present study, we characterized the CSFV in porcine kidney cells. The CSFV vaccine strains showed enhanced replication following fifteen passages in porcine kidney cells. Nucleotide sequence analysis of E2 protein gene of cell culture adapted vaccine strain of CSFV showed a mutation in putative amino acid sequences that are identical to its virulent counterpart. The study suggests the possibility of exaltation in vaccine strains following its adaptation in host cells and paves the way for further exploring the biology of its outbreak.

The physical characterization of CSFV vaccine at different temperature, pH, and saline solutions imparted an idea regarding the stability of vaccine at different physical conditions and solution. Study showed the stability of CSFV up to 56ºC and pH range 6 to10. The stability of CSFV significantly decreased above 72°C and in high acidic pH. In addition, CSFV was found unstable at saline solution at 4°C after 72hrs. The result will be useful in formulating the storage condition, pH, and buffers for storing the lapinized CSFV vaccines without having any deterioration in its efficiency and viability.

2.2 Introduction

Recent reports on CSFV outbreaks in India suggest that the mean prevalence of its antibodies in suspected sera was 63.3%. Moreover, 76.7% of the suspected samples were found positive for CSFV antigen, which indicate that the disease is endemic to India (Nandi, Muthuchelvan et al. 2011). Vaccination is the best way to control CSFV infection in swine, however, outbreaks have been reported in many areas, due to improper vaccination and faulty management practises (Suradhat and Damrongwatanapokin 2003, Ji, Niu et al. 2014). Chinese strains and lapinized vaccine are effective in controlling

CSFV infection since 1960 from domestic pigs. Both Chinese strain of CSFV has been further adapted into many commercial vaccine strains (Luo, Li et al. 2014)

Adaptation of virus is a general procedure in lab through cell culture for its further characterization and study, because the adapted virus is able to culture or amplify easily. Adapted virus can go through many passages during the research work and any advance study like protein expression, structure analysis, genome manipulation, or mutation and recombinant technology can be studied or performed. Here virus was adapted and molecular characterization was done. The lapinized vaccine was studied for physical stability.

2.3 Materials and methods

The summary of methodology is presented as pictorial form in Figure: 2.1

2.3.1 Cell and viruses

The porcine kidney cells (PK-15) were procured from the ATCC (Manassas, VA). The cells were maintained in Eagle's minimal essential medium (EMEM) containing 10% fetal calf serum (Invitrogen, Grand Island, NY) tested free for BVDV at 37°C in 5% CO2. Three lapinized vaccine viruses, namely IVRI (Indian classical strain), Assam lapinized (Assam), and virus isolated from vaccinated pigs (local strain), were procured from the Department of Microbiology, College of Veterinary Science, Khanapara, Guwahati, India. The sequences of the three lapinized vaccine strains regularly used under field conditions were extracted from GenBank (accession numbers) EU857642 (Indian classical strain), EU567078 (Assam strain), and KP195022 (local strain).EU857642 (Indian classical strain), EU567078 (Assam strain), and KP195022 (local strain).

2.3.2 Passaging or Adaptation of viruses

The CSFV strains were passaged in PK-15 cells in 80% to 90% confluence blindly for 15 passages. For CSFV infection in PK-15 cells, 1 ml of virus inoculum treated with antibiotic and filtered through a 0.25-µ syringe filter was allowed to adsorb on the monolayer for 60 min. The EMEM containing 5% fetal calf serum tested free of pestivirus was overlaid after draining the virus inoculums from PK-15 cells. The cells are further incubated at 37°C under 5% CO2 for 5 d. The infected PK-15 cells were freeze-

thawed for three cycles and clarified at 3000 rpm, and the supernatant was collected as virus inoculum for the second passage and stored at −80°C.

2.3.3 Immunoperoxidase or Immunofluorescence

The replication of CSFV strains in the PK-15 cells was analyzed by immunofluorescence (IF) and immunoperoxidase (IP) staining using the WH303 monoclonal antibody as described previously (Wensvoort, Terpstra et al. 1986). The CSFV titers in 50% endpoint tissue culture infectious dose (TCID50) units/milliliter were determined on monolayers of PK-15 cells using the Reed and Muench method (Hierholzer JC and Killington 1996). The CSFV Indian classical strain, Assam strain, and local strain having a titer of $10^{3.25}$, 10^{2}, and $10^{2.80}$ TCID50/ml, respectively, were used for their first passage in PK-15 cells.

2.3.4 RNA Extraction and RT-PCR

Viral RNA was extracted from supernatants of infected cells by TRIzol (Invitrogen) using a standard protocol. Further, cDNA was prepared using a gene-specific reverse primer designed on the basis of the whole genome sequence of the CSF strain: E2 rev (5′-CCG CTC GAG ACC AGC GGC GAG TTG-3′) followed by PCR using E2 rev and E2 for (5′- CCC AAG CTT CGG CTA GCCTGC AAG G-3′) primers. The PCR amplified products were sequenced directly using a BigDye terminator v 3.1 matrix standard kit and 3130xl Genetic Analyzer data collection software v3.0 (Applied Biosystems Inc). The E2 gene was sequenced at least three times from three independent RNA preparations to ensure a consensus sequence.

2.3.5 Phylogenetic analysis and amino acid alignment between passage and wild (parental) strain

Phylogenetic analysis of the E2 gene sequence of all the three strains with available GenBank sequences was performed by the Molecular Evolutionary Genetics Analysis software (MEGA4) using the maximum parsimony method. The robustness of the groupings in the neighbor-joining analysis was assessed with the 1000 bootstrap value (Kumar, Nei et al. 2008). The secondary structure of the putative E2 protein sequences was analyzed by PSIPRED software (http://bioinf.cs.ucl.ac.uk/psipred/).

Amino acid sequence alignment was performed by ClustalW multiple alignment algorithm of the MegAlign program of the DNASTAR Lasergene 7 software package.

GenBank accession number NC_002657 has been taken as reference sequence for CSFV. The percentage identity of nucleotide and amino acid was performed by same software of DNASTAR

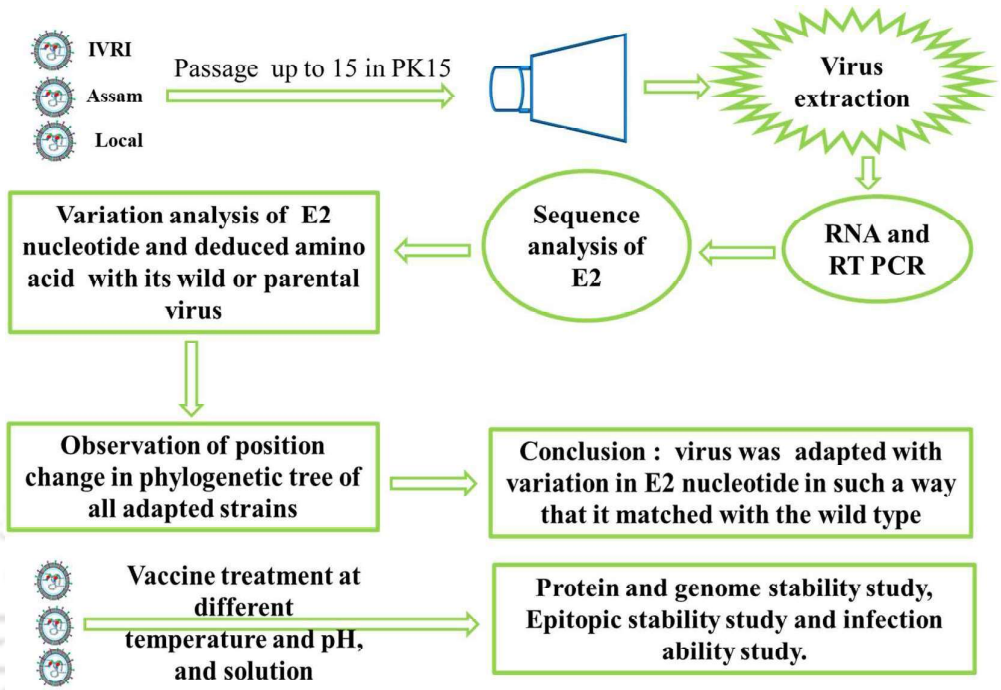


Figure: 2.1 Graphical representation of the used methodology

2.3.6 Vaccine virus characterization at different physical condition

The CSFV suspension was prepared by dissolving 0.1mg of lyophilized virus in 1 ml of phosphate buffered saline (PBS). The stability of CSFV proteins was observed by treating the sample at different conditions of pH, temperature and salt concentrations. Sample for temperature study was prepared by diluting 1 µl of vaccine suspension in 30 µl of PBS, and keeping at temperatures: RT, 37°C, 42°C, 56°C, 68°C, 72°C and 96°C with appropriate control for 3 hours. Samples for pH study were prepared as previously described, with different pH of the solutions: pH 2, 4, 6, 7, 8, 10 and 12; for 3 hours at room temperature (Rani, Gogoi et al. 2014). Stability of CSFV proteins was studied by preparing CSFV samples in 0.4% NaCl and 0.9% NaCl solution at 4°C for 12, 24, 36, 48, 72 and 96 hours. The samples after treatment were loaded on 12% SDS gel after staining with Coomassie Brilliant blue dye. The pH and temperature treated samples were coated

onto polystyrene 96-well plate and analyzed by spectrophotometer as described earlier (Kumar, Barman et al. 2016).

The replication of CSFV in the PK-15 cells was analyzed by immunofluorescence (IF) and immunoperoxidase (IP) staining using the monoclonal antibody as described above.

The stability of genomic RNA of CSFV was determined by RT-PCR using E2 gene specific primers. The viral RNA from all the samples were isolated using TRIzol reagent (Invitrogen, USA) following standard protocol. The cDNA was prepared by random hexamer primers followed by its PCR using E2 specific forward and reverse primers (E2For-5′-AGGAGAGACAAGCCCTTTCC-3′, E2Rev-5′-TTCACACATGTCC AGTTGCC-3′).

2.4 Results

2.4.1 Immunofluorescence and Immunoperoxidase

The infection of CSFV vaccine strains did not show any cytopathic effect in PK-15 cells up to 15 passages. However, the CSFV strains showed positive IF and IP staining using the WH303 monoclonal antibody (Figure: 2.2). The CSFV Indian classical strain, Assam strain, and local strain showed a titer of $10^{7.25}$, 10^{5}, and 10^{6} $TCID_{50}$/ml, respectively, after 15 passages.

2.4.2 RT-PCR of E2 gene

CSFV after each passage was confirmed by RT-PCR analysis using E2 gene specific primers for the respective strains. (Figure: 2.2E). The E2 gene sequences of all three vaccine strains following 15 passages were analyzed for their putative amino acid sequences.

2.4.3 Phylogenetic, nucleotide and amino acid analysis between passage and wild (parental) strain

The nucleotide sequence of the E2 gene of the PK-15-adapted Indian classical strain showed 93.6% identity with its parental strain. Similarly, the nucleotide sequence of the E2 gene of the local strain and Assam strain showed 99.8% and 99.9% identity, respectively, with their parental strain after its adaptation in PK-15 cells (Table. 2.1). The putative amino acid sequence of the E2 protein of the Indian classical strain and local

strain of CSFV showed 95.6% and 99.4% identity, respectively, with the parental strain after its passage in PK-15 cells (Table. 2.1). Phylogenetic analysis of the E2 protein of cell culture-adapted vaccine strains with different CSFV strains showed clustering of the Indian classical strain with the local lapinized vaccine (Figure: 2.3). A comparison of the amino acid sequences between the Indian classical strain and the reference sequence of the E2 protein of CSFV (NC_002657) showed differences of K720E, V744I, T886A, I971T, and A975E (Figure: 2.4). The conserved motif 829TAVSPTTLR837 in domain A was found identical in all the strains independent of its number of passages in PK-15 cells (Lin et al. 2000). The amino acids 745I, 763S, 764L, 864G, 917V, and 940D were found unique to the virulent strain and were absent from the vaccine strains (Figure: 2.4). The CSFV strain Assam does not show any difference in amino acid level after its subsequent passage in PK-15 cells (Figure: 2.4). The local lapinized vaccine showed the mutation of N181K and W182L following its adaptation in PK-15 cells.

2.4.4 Effect of physical conditions on vaccine virus

The CSFV treated at different temperatures showed degradation of viral proteins at 72^0C (Figure: 2.5 A and B). All the experiments were repeated thrice independently in order to get a consensus result. The CSFV vaccine treated at various pH showed degradation of viral proteins. The extreme acidic (pH=2-4) and alkaline conditions (pH>10) are detrimental to the viral proteins, suggesting the importance of pH on its viability (Figure: 2.5D and 2.5E). The ELISA reading of the CSFV treated at various temperature showed reduced titer following 56^{o}C (Figure: 2.5C). Surprisingly, CSFV treated at various pH showed similar ELISA titer (Figure: 2.5F). The CSFV kept under buffer containing 0.9% NaCl showed slight degradation following 72h post treatment while the same kept in 0.4% NaCl showed more degradation of protein (Figure: 2.6). The infectivity assay of temperature and pH treated CSFV vaccine samples were analysed by immunofluorescence assay in PK-15 cells. The temperature treated CSFV samples showed decreased infectivity above 56^{o}C (Figure: 2.7). In pH treated sample the infection efficacy showed decrease in high acidic condition and at pH 6 to 10 showed similar fashion of infection efficacy (data not shown). The RNA genome of CSFV showed stability at wide range of temperature while the same showed degradation at acidic pH condition (Figure: 2.8).

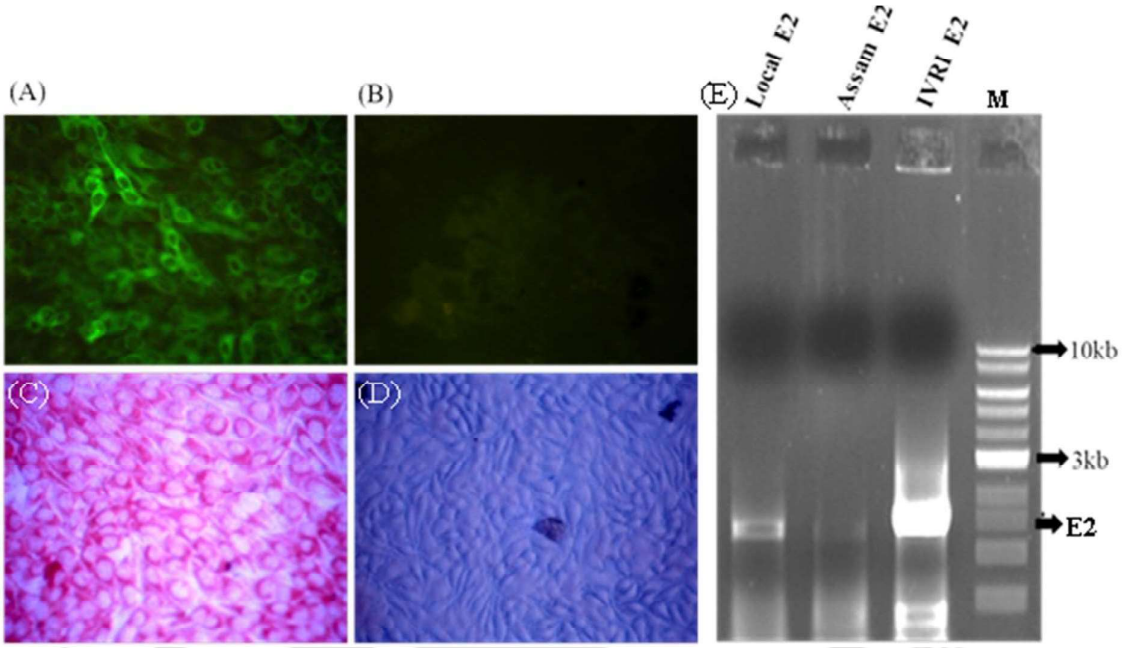


Figure: 2.2 Immunofluorescence and immunoperoxidase staining of the classical Indian strain of CSFV following its infection in PK-15 cells. The infected cells showing positive fluorescence when stained with the WH303 monoclonal antibody followed by the FITC conjugated secondary antibody (A). No fluorescence was observed in uninfected cells (B). Similarly, positive peroxidase staining was observed when horseradish peroxidase-conjugated secondary antibodies were used to stain the infected cell (C). No peroxidase staining was observed in uninfected cells (D). Amplification of E2 gene from three different strains of CSFV and M is marker (E).

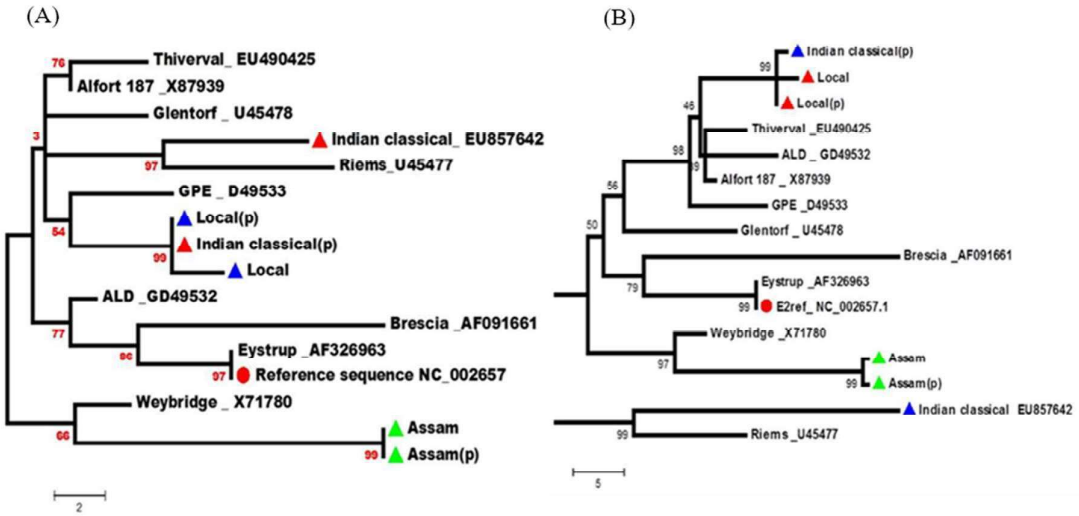
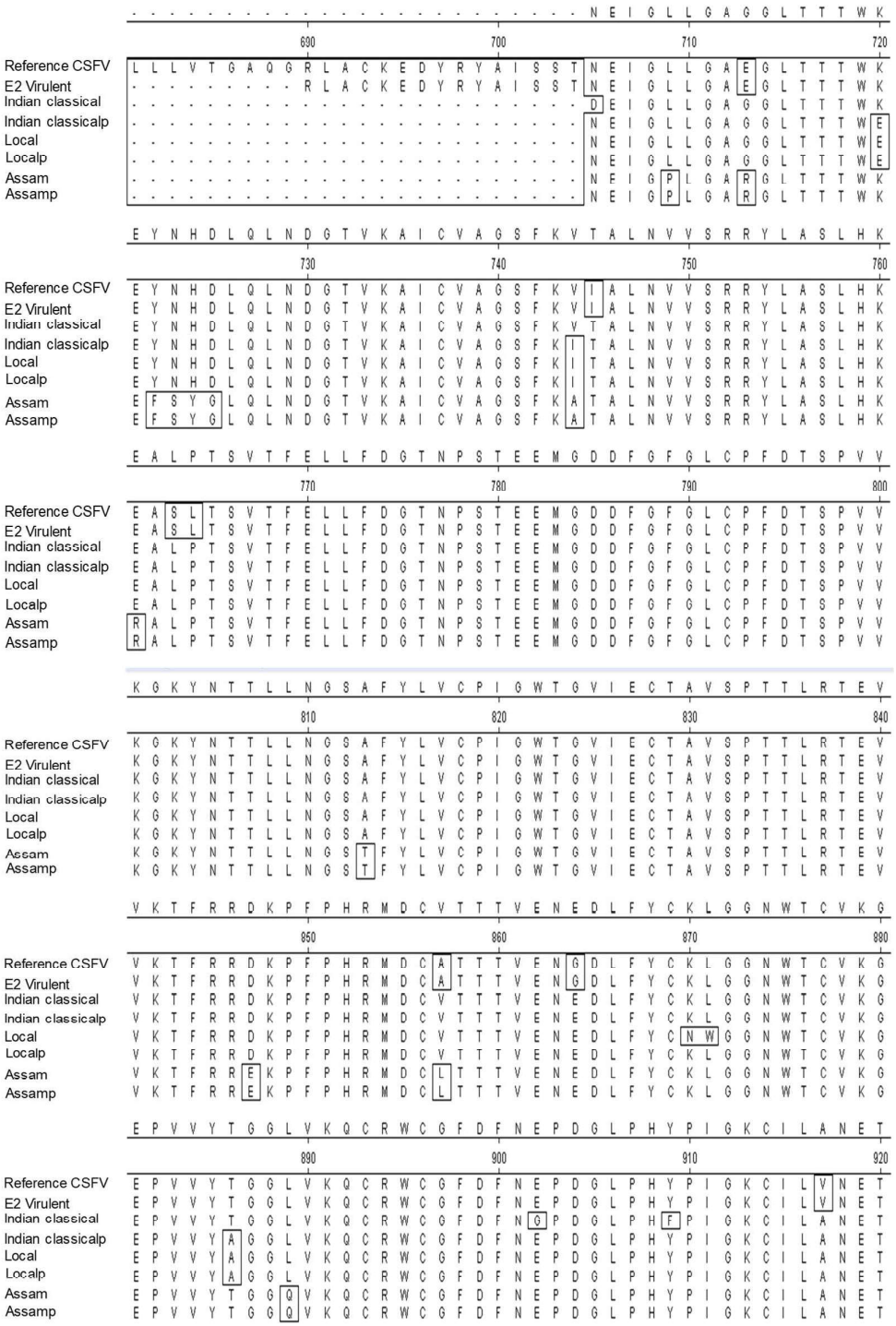


Figure: 2.3 Phylogenetic analyses of passaged (adapted) viruses. Tree showing the position of viruses on the basis of nucleotide sequences of E2 gene (A) and on the basis of amino acid sequence of E2 protein gene of CSFV (B).

Nucleic Acid						
	Indian classical	Indian classicalp	Local	Localp	Assam	Assamp
Indian classical		93.6	93.8	94.0	93.8	93.7
Indian classicalp	95.6		99.4	99.6	95.1	95.0
Local	95.0	99.4		99.8	95.3	95.2
Localp	95.6	100	99.4		95.5	95.4
Assam	93.3	94.4	93.9	94.4		99.9
Assamp	93.3	94.4	93.9	94.4	100	
			Amino Acids			

Table. 2.1 The nucleotide sequence and amino acid identity of E2 protein gene of different strains of classical swine fever virus (subscript "p" indicates the passaged virus).


Reference CSFV
E2 Virulent
Indian classical
Indian classicalp
Local
Localp
Assam
Assamp

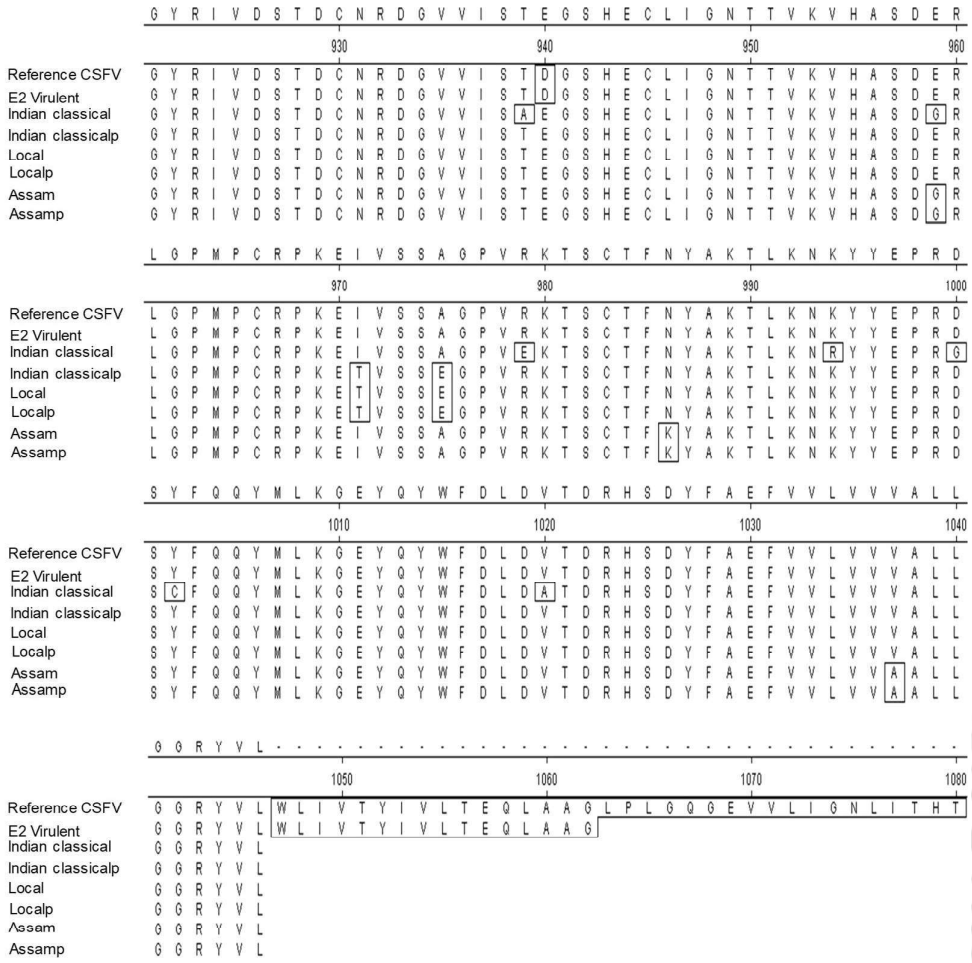
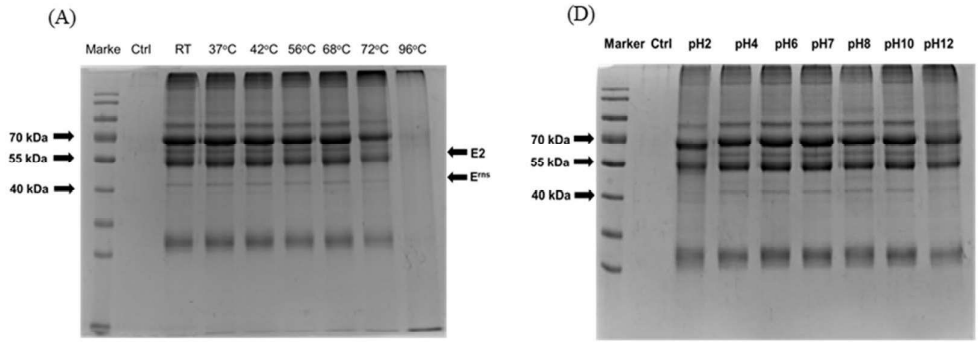


Figure: 2.4 Amino acid sequence comparison of classical swine fever virus (CSFV) vaccine strains following its passage in porcine kidney cells (p indicates passaged virus). Amino acid sequence alignment was performed by ClustalW multiple alignment algorithm of the MegAlign program of the DNASTAR Lasergene 7 software package. GenBank accession number NC_002657 has been taken as reference sequence for CSFV. The E2 protein sequence encoded by the reference strain was listed as "E2 virulent" for the alignment in order to depict the correct amino acid position with respect to the CSFV polyprotein.



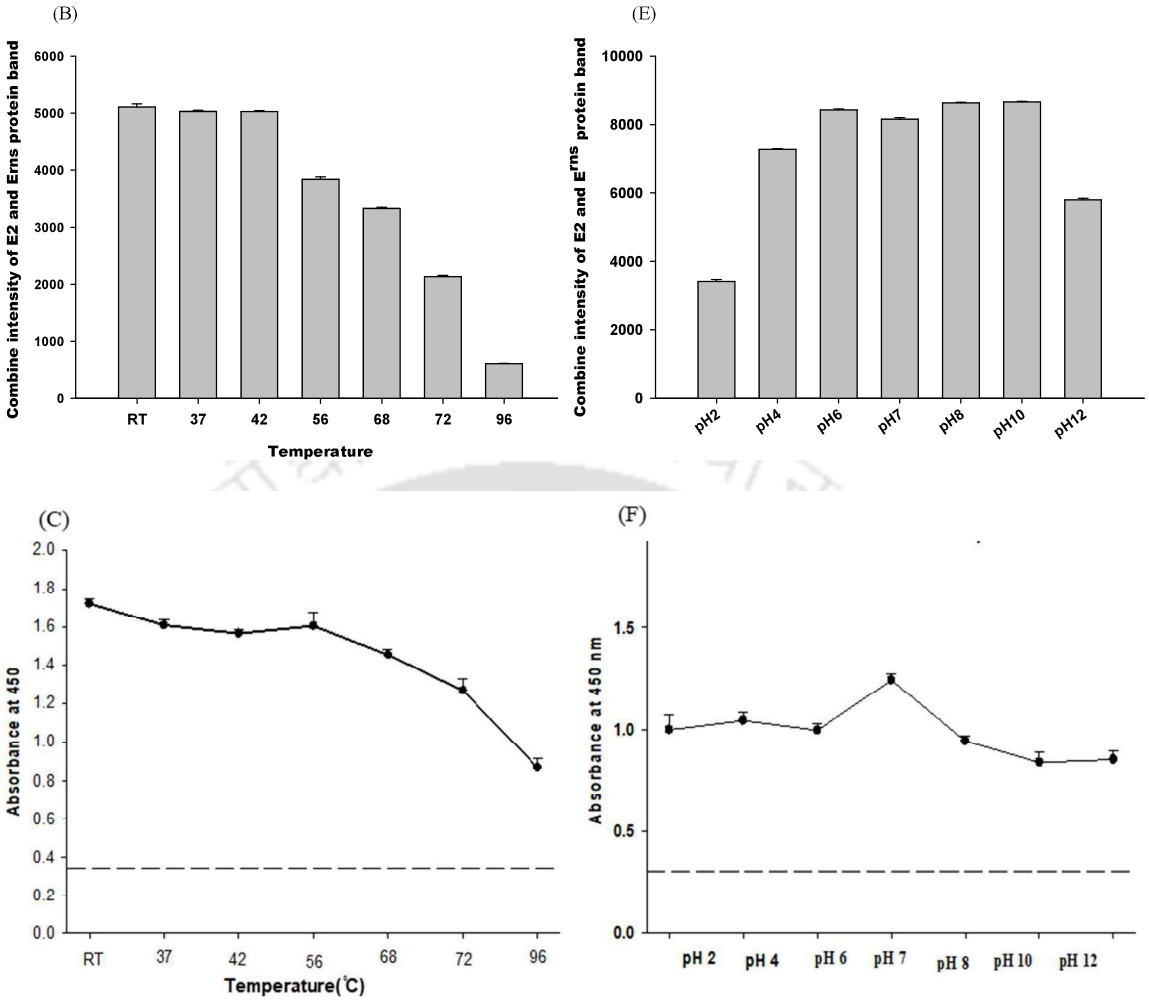
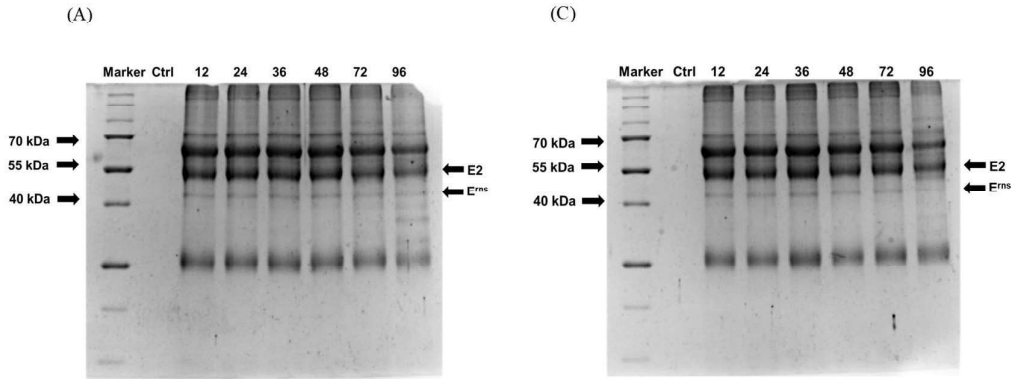


Figure: 2.5 Stability of CSFV proteins at different temperature and pH. SDS PAGE analysis of CSFV treated at different temperature (A), combine intensity of E2 and E^{rns} proteins after different temperature treatment (B), ELISA reading of temperature treated CSFV (C). Similarly, SDS PAGE of CSFV treated at different pH (D), combine intensity of E2 and E^{rns} proteins after different pH treatment (E), ELISA reading of pH treated CSFV showing stability of immunogenic protein (F).



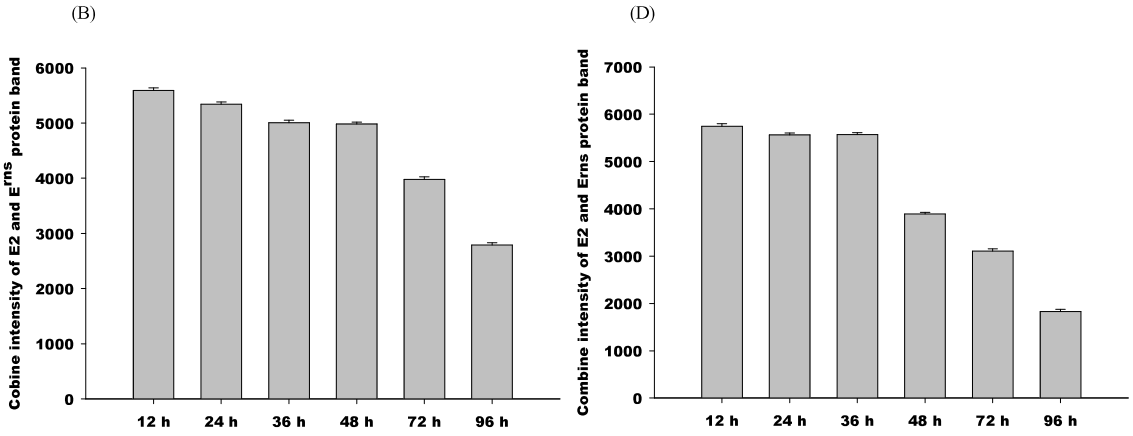


Figure: 2.6 Stability of CSFV proteins at different saline concentration. SDS PAGE analysis of CSFV proteins at 0.9 % NaCl (A), histogram of combine band intensity of E2 and E^{rns} proteins after treatment with 0.9 % NaCl showing significant degradation of proteins at 96 hrs (B), SDS PAGE of 0.4 % NaCl treated CSFV(C), combine intensity of E2 and E^{rns} proteins after treatment with 0.4 %NaCl (D) **.**

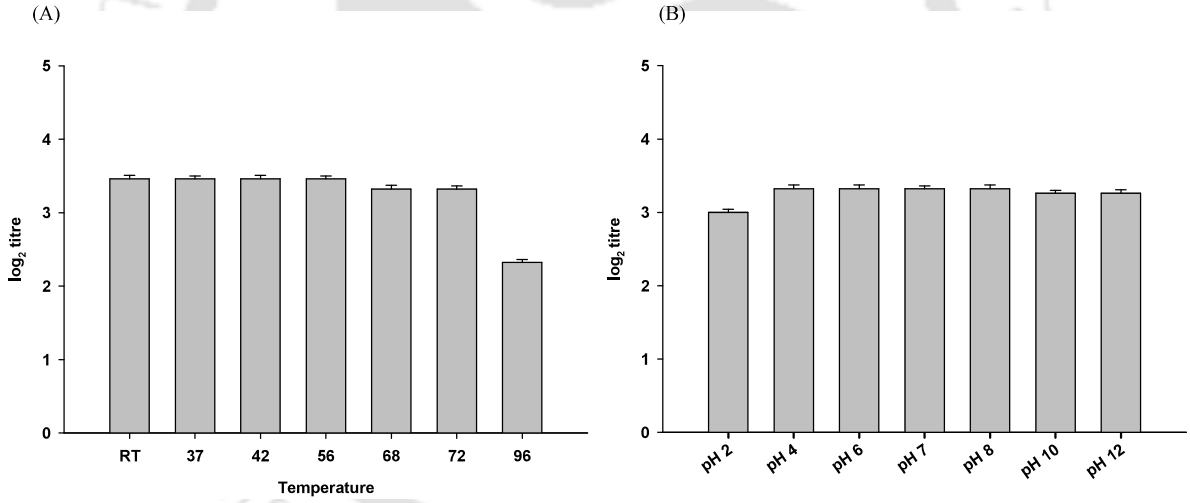


Figure: 2.7 Infectivity of CSFV after thermal and pH treatment. Histogram showing the infectivity of CSFV in log_2 titer. CSFV showing reduced infectivity from 68 °C to 96 °C (A), while infectivity of CSFV at different pH seems constant (B).

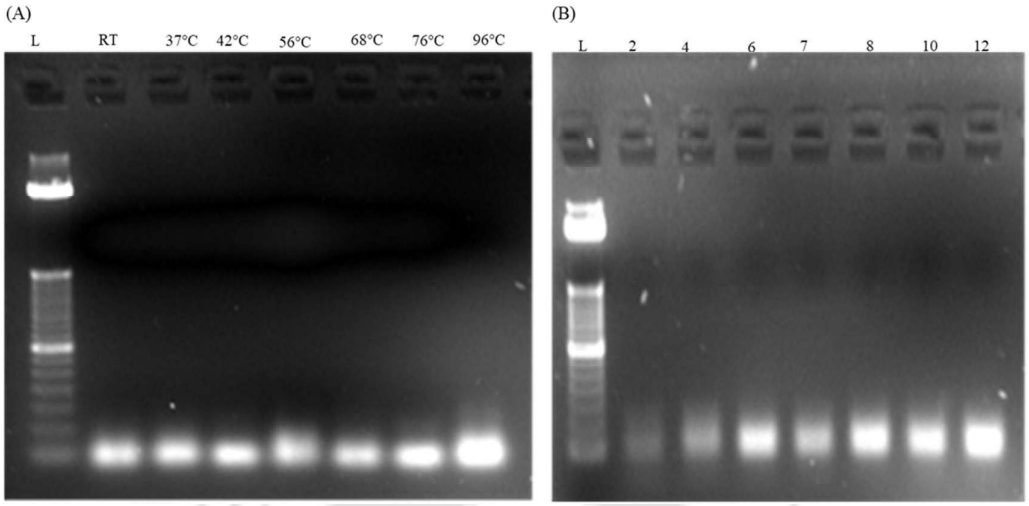


Figure: 2.8 RT-PCR of partial E2 after temperature and pH treatment. CSFV genome was found stable at wide range of temperature (A). CSFV genome showing degradation at lower pH (B).

2.5 Discussion

Our result showed that these amino acids were unchanged even after 15 passages suggesting its specificity for the virulent CSFV strains. How these specific changes contribute to the altered phenotype observed for the CSFV E2 protein gene remains to be determined. However, it has been shown recently that two single amino acid changes, S763L and P968H, in the E2 protein can cause the attenuation of CSFV in swine (Fahnoe, Pedersen et al. 2014). In addition, it has also been shown that T830A substitution in the E2 protein of CSFV can increase the pathogenicity (Tamura, Sakoda et al. 2012). Secondary structure analysis of the E2 protein of the PK-15- adapted Indian classical, local, and Assam strains showed the helix towards its carboxy terminus while the amino terminus is dominated by strands and coils similar to their non-passaged forms. Our results of the cell culture adaptation of CSFV corroborate the earlier report where it has been shown that structural proteins can mutate faster than other viral proteins in order to selectively escape the host immune system. (Kwang, Littledike et al. 1992) Moreover, the adaptation of CSFV in PK-15 showed subsequent high infectivity at a lower TCID50 titer suggesting its better binding efficiency with the host cell surface receptor. A monoclonal antibody used in the study is targeted to the conserved amino acid sequences 829TAVSPTTLR837 and reacts equally with all three strains of CSFV. Our finding corroborates the earlier report where a change in amino acid sequences in the E2 protein contributed to the better binding efficiency of CSFV to the cell surface receptor (Liang, Sainz et al. 2003). The conserved amino acid sequences 829TAVSPTTLR837 in all the

CSFV strains as suggested earlier as linear epitope might not be important in the adaptation of the virus in vitro (Lin, Lin et al. 2000, Qi, Liu et al. 2008). The E2 protein is supposed to be a major determinant of virulence in CSFV, and changes in some critical amino acids might modulate its virulence (Risatti, Borca et al. 2005, Tamura, Sakoda et al. 2012). The existence of a conserved amino acid sequence in all three strains and their reactivity towards theWH303 monoclonal antibody suggest the possession of the immunogenic epitope in all three CSFV vaccine strains. However, the evaluation of cross-neutralization capacity in primary host sera with other vaccine strains and currently circulating wild strains can give the status of full protection in vaccinated animals. The study will be useful to understand the biology of CSFV and its repeated outbreaks in the vaccinated flocks. We have limited information about the biology of CSFV, and there are strains out there that can behave differently than what is taken as dogma. Further, exploring the mutations in the E2 protein gene of CSFV by reverse genetics will help us to modulate or attenuate the virus for the development of a better live recombinant vaccine.

. The failure of vaccination and the emergence of its virulent pathotypes are the major causes of CSFV outbreaks in field condition. The CSFV outbreaks are most visible in countries, where climate is hot and maintaining a cold chain for the vaccine formulation is a daunting task in field condition. In the present work CSFV showed susceptibility towards the temperature and pH suggesting its sensitivity towards the elevated temperature and extreme conditions of pH. The findings were further supported by reduced ELISA titer suggesting the degradation of the native confirmation of viral proteins. However, the similar effect was not appreciated in the CSFV treated at different pH suggesting the intactness of the epitope of its surface proteins. CSFV has RNA as a genetic material and its biology largely depends on the integrity of its surface glycoproteins. It may be possible that the thermal stability of CSFV genome might depend on duration of experiment and also on protection by structural proteins like C protein directly and indirectly through other viral proteins. In addition to its surface glycoproteins, other proteins also play an important role in the pathogenicity of CSFV in pigs.

Our finding is different than the other enveloped RNA viruses, where pH and solvent play a minimal role in their stability (Rani, Gogoi et al. 2014, Rani and Kumar 2015). The CSFV treated at different temperature showed reduction in its infectivity in PK-15 suggesting the degradation of its surface glycoproteins. It has been shown that

infectivity of the CSFV depends largely on its surface glycoproteins (Wang, Nie et al. 2004, Wu, Li et al. 2016). The surface glycoproteins of an enveloped virus interact with the host cell receptor making it permissible for its infectivity.

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Chapter III

Development of single serum ELISA: using prokaryotic expressed protein E2 of CSFV

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Chapter III

Development of single serum ELISA: using prokaryotic expressed protein E2 of CSFV

3.1 Abstract

One major drawback of diagnostic based on ELISA is that high dilutions of serum might result in false negatives and low dilutions of serum might result in false positives. The virus surface protein E2 is the major immunogenic protein eliciting protective immunity against CSFV infection in swine. The whole virus antigen cannot differentiate CSFV from other pestiviruses as it cross reacts with border disease and bovine *viral* diarrhoea *viruses*. Commercial available ELISA is based on the whole CSFV particle and can lead to false positive results. Moreover, the available commercial ELISA is not cost effective. In the present study, a recombinant E2 protein based single serum dilution ELISA was developed which showed enhanced sensitivity, specificity, and accuracy as compared to commercial CSFV detection ELISA. The recombinant E2 protein based ELISA could be an alternate to existing diagnostics against CSFV infection in pigs.

3.2 Introduction

E2 is an envelope glycoprotein present on the surface of CSFV and is important to induce host immune response during infection (Zhang, Yu et al. 2006, Qi, Zhang et al. 2009). The E2 protein contains conserved antigenic determinant regions and it is the major immunogenic protein eliciting protective immunity against CSFV infection in swine (Greiser-Wilke, Moennig et al. 1990, Rumenapf, Stark et al. 1991, van Rijn, Bossers et al. 1996). The E2 protein contains two Linear B cell epitopes YYEP and TAVSPTTLR spanning towards its carboxy and amino terminus, respectively. The amino acid sequence motif YYEP is specific to pestivirus while TAVSPTTLR is specific to CSFV (Yu, Wang et al. 1996, Lin, Lin et al. 2000). In addition, E2 protein is accompanied with four relatively independent antigenic domains (A, B, C, and D). The domain A has three subdomains (A1, A2, and A3). The E2 is frequently used to design DNA vaccines against CSFV by different research groups (Bouma, de Smit et al. 1999, Beer, Reimann et al. 2007). Structurally, the E2 protein forms a homodimer during entry and heterodimer with E1 while attachment of the viral particles to the cell (Zhang, Yu et al. 2006). In epidemiological surveys, detection of virus-specific antibodies in serum

samples is important in order to monitor the circulation of wild CSFV in population. Neutralizing assay is the test of choice to detect CSFV infection in the laboratory. However, detection of neutralizing antibody is time- consuming, needs skill and well setup cell culture laboratory. Development of single dilution indirect ELISA can be a convenient alternative tool to detect CSFV specific antibody in pig sera (Yang, Li et al. 2012, Li, Mao et al. 2013) using complete E2 protein as detecting antigen. Single serum dilution could be better than serial dilution method because it requires less time, chemical and plastic ware making it a cost-effective assay. Here we are reporting the expression of the complete E2 protein using the bacterial expression system. The bacterial expressed E2 protein showed efficient binding with monoclonal antibody and with the serum collected from the field outbreaks. The study will be useful in designing an efficient diagnostics against CSFV infection.

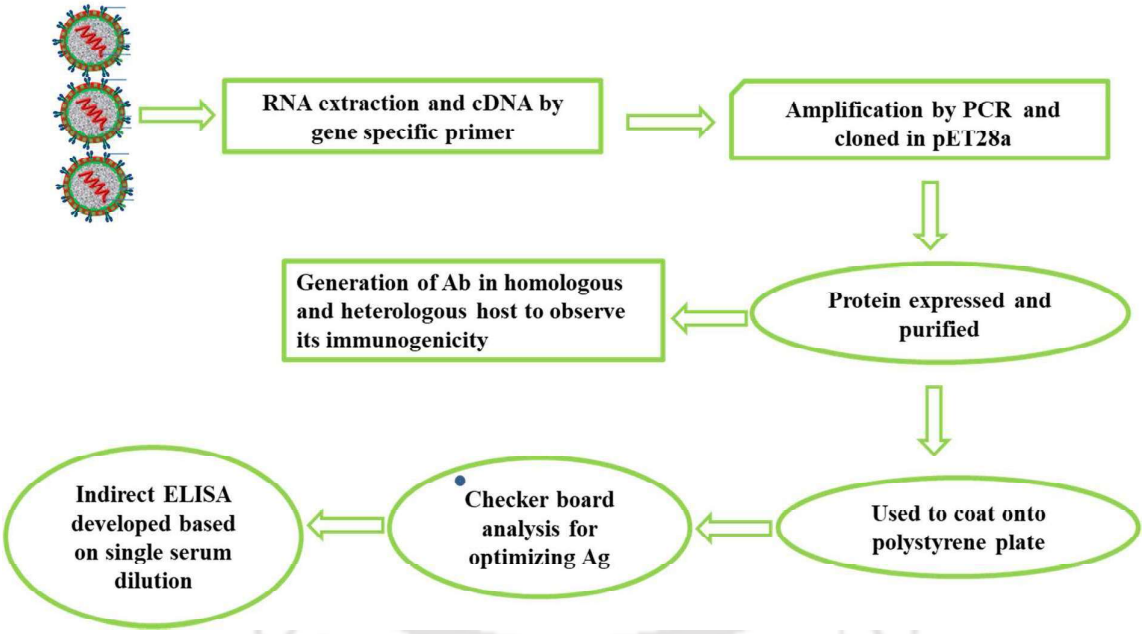

Figure: 3.1 Graphical representation of the used methodology

3.3 Materials and methods

3.3.1 Cell and viruses

The porcine kidney cells (PK-15) were procured from ATCC (Manassas, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and essential antibiotics at 37◦C in 5% CO2. The CSFV vaccine strains were procured from a college of veterinary science, Guwahati, India. The virus was grown in PK-15 cells using standard protocols (Hulst, van Gennip et al. 2000). The

infectious virus particles were recovered by repeated freeze and thaw followed by filtering the extracted supernatant through the 0.22µm membrane filter. The stock of the virus was stored at −80◦C for subsequent use. Titration of the virus was done in 96-wells micro-titer plate containing PK-15 cells. Vaccine virus was diluted ten-fold in DMEM containing 10% calf serum into each of the five wells in a 6-well plate. The titer of virus stock was calculated by immunoperoxidase assay using CSFV monoclonal antibody after 72 h post-infection as described earlier (9880012).

3.3.2 RNA extraction and RT-PCR

Viral RNA was extracted using TRIzol (Invitrogen, USA) according to the standard protocol. The cDNA was synthesized from extracted RNA using Superscript II RT(Invitrogen, USA) and gene-specific reverse primer (5'-CCGCTCGAGTCAAC CAGCGGCGAGTTGTTCTG-3') designed from available GenBank sequence (Accession number NC 002657). The E2 gene sequence was PCR amplified using E2 forward (5'-CGGAATTCATGCGGCTAGCCTGCAAGGAAGATTAC- 3') and E2 reverse (5'-CCGCTCGAGTCAACCAGCGGCGAGTTGTTCTG- 3') primers. Under-line sequences are complementary to the CSFV genome and italics sequences are the restriction site *EcoR*I and *Xho*I, respectively. The amplified PCR products were purified by QIA quick gel extraction kit (QIAGEN, Germany) and sequenced by Big Dye terminator v 3.1matrix standard kit and 3130xl Genetic Analyzer Data Collection software v3.0 (Applied Bio -systems, USA). The data extracted from analyzer was analyzed by DNA star software (DNASTAR Inc., USA).

3.3.3 Cloning, expression and purification of the E2 protein

The complete E2 protein gene was cloned into pET28a prokaryotic expression vector (Novagen, Germany) flanking *EcoR*I and *Xho*I restriction sites. The pET28a has His tagged at the N terminal which was used to purify the E2 protein by affinity chromatography. The integrity of the E2 gene after cloning was confirmed by sequencing. The pET28a containing complete E2 gene was transformed into Escherichia coli BL21 (DE3) pLysS cells (Novagen, Germany) and induced by 1 mM isopropyl-D-thio-galactoside (MBI Fermentas, Germany). The fusion protein was extracted after induction of transformed BL21 cells for 4 h at 37◦C and purified by affinity chromatography containing Ni-NTA (Invitrogen, USA). 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 dialyzed using 10% glycerol in PBS. The concentration of the purified protein was determined by modifying Lowry's Protein assay kit according to the manufacturer's protocol (Pierce, USA).

3.3.4 Immunoblot of purified E2 protein and *Immunization study*

The expression of the recombinant E2 protein was further confirmed by SDS-PAGE and western blot using an anti E2 monoclonal antibody WH 303 (Wensvoort, Terpstra et al. 1986). Two rabbits were immunized subcutaneously with 0.5 mg of purified recombinant E2 protein emulsified in Freund's complete adjuvant (Sigma, USA) for the preparation of polyclonal antibodies. Subsequent booster was given by purified recombinant E2 protein with Freund's incomplete adjuvant (Sigma, USA) for 2 occasions at 14 days interval. The sera sample was collected 14 days after final injection and stored at −80◦C. The antibody against recombinantE2 protein was confirmed by western blot using goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, USA) as well as by commercial CSFV kit (HerdChek, IDEXX, USA). A total of 210 pig serum samples were screened for the CSF infection. Blood samples of suspected pigs were collected under the aseptic condition and serum was separated after centrifugation at 1500 × g for 20 min, and stored at −20◦C until use. Additionally, serum samples were screened negative for swine flu and porcine reproductive and respiratory syndrome using a commercial kit (PRRS X3 IDEXX, USA).

3.3.5 Development of a protocol for Indirect ELISA

96-well flat bottom polystyrene plates (Greiner, USA) were coated with recombinant E2 protein (3 µg/well) using nitrate buffer (pH = 9.6) and incubated at 4◦C overnight. Plates were washed with phosphate buffer saline containing Tween-20 (PBST) and blocked with 5% lactalbumin hydrolysate for 1 h at 37◦ C. Optimal concentration of E2 protein was determined by the checkerboard titration method. Pig serum samples were initially diluted 10 times and then diluted serially and incubated at 37◦C for 1 h. The plates were then washed with PBST, and incubated with 100µl of the HRP-conjugated anti-pig antibody raised in rabbit (Pierce, USA) for another 1 h at 37◦C temperature. The plates were washed and the E2 protein binding with serum samples was detected with 100µl of TMB (Invitrogen, USA) for 15 min at 37◦C temperature. The enzymatic reaction was stopped by 100µl of 2 M H2SO4, and plates were read at 450 nm in a micro-titer plate reader (Biotek, USA). Any serum sample showing an OD above the mean +3

standard deviation of the negative wells was considered positive. The negative serum samples were used to construct positive-negative threshold (PNT) baseline as shown earlier (Snyder, Marquardt et al. 1983). Similarly, the positive curve was also plotted. The absorbance of the test sample dilutions was calculated using the formula:

SP ratio = (OD of sample – OD of negative)/ (OD of positive – OD of negative)

The PNT line was calculated using negative serum samples which were screened negative by a commercial kit. The collected negative serum samples were diluted and resultant OD values were plotted against dilution. The resultant PNT line was used to find out the titer for the known positive serum samples by the subtraction method as described earlier (Snyder, Marquardt et al. 1983). The OD values obtained for every logarithmic dilution was compared with observed titer and the highest correlation coefficient was selected to calculate the titer from that dilution. The constants like slope and intercept were calculated by the scatter plot as described earlier (Snyder, Marquardt et al. 1983). The sensitivity, specificity, and accuracy of the single dilution sera in comparison to the commercial CSFV diagnostic are deter-mined using following formulae.

Sensitivity = $(x/x + y) \times 100$

where 'x' is the number of sera positive by commercial CSFV diagnostic ELISA and single dilution ELISA; 'y' is the number of sera negative by our test ELISA and positive by commercial CSFV diagnostic ELISA.

Specificity = $(\alpha/ \alpha + \beta) \times 100$

Accuracy = $(x + \alpha/x + y + \alpha + \beta) \times 100$

where ' α' is the number of sera negative by commercial diagnostic ELISA and single dilution ELISA; ' β' is the number of sera positive by single dilution ELISA and negative by commercial diagnostic ELISA.

3.4 Results

3.4.1 Cloning, expression and purification of the E2 protein

The PCR amplified product of CSFV E2 protein was successfully cloned into pET28a vector and showed a release of the desired fragment upon digestion with *EcoR*I and *Xho*I. Furthermore, the sequencing of the E2 protein showed intactness of the

nucleotide sequence. The recombinant E2 protein expressed in BL21 cells as a fusion protein with His tag was about 56 kDa in size (Figure: 3.2). The average yield of the recombinant E2 protein was 70 mg/l of the bacterial culture used for its purification. The recombinant E2 protein showed the corresponding band of 56 kDa in western blot using raised polyclonal rabbit sera (Figure: 3.2). The western blot results and indirect immunoperoxidase assay confirmed the specificity for raised polyclonal antibodies.

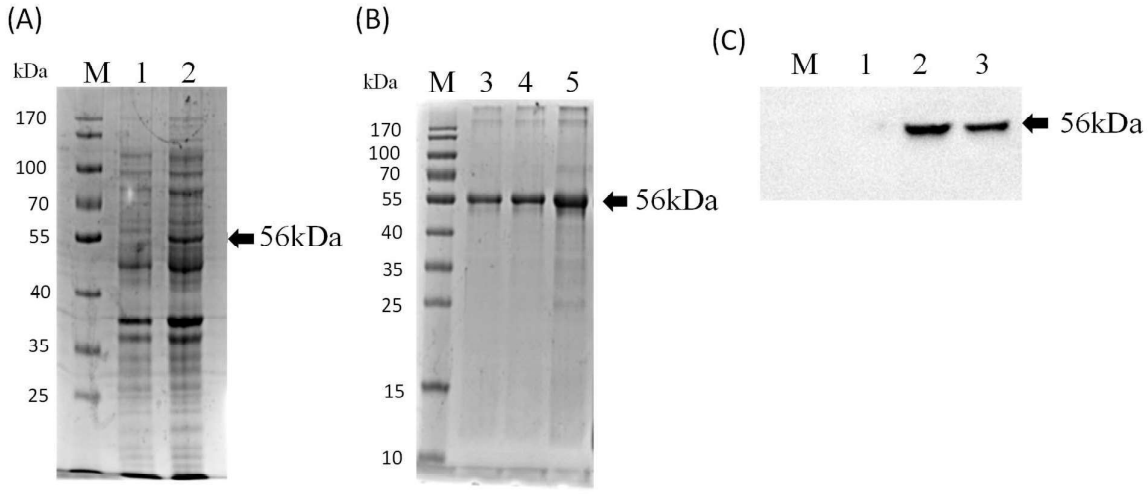

Figure: 3.2 Expression analysis of recombinant E2 protein of classical swine fever virus (CSFV). The SDS-PAGE analysis of the E2 protein of CSFV expressed in pET28a prokaryotic expression vector. M: protein molecular weight marker; Lane 1: uninduced recombinant pET28a containing E2 protein gene; lane 2: expression of protein in recombinant pET28a containing E2 protein gene after 2 h of 0.5 mM IPTG induction (A). Lane 3, 4, and 5: different fractions of purified recombinant E2 protein (B). Western blot analysis of recombinant E2 protein using polyclonal antibody raised in rabbits. Lane1: pET28a vector control; lane2: purified recombinant E2 protein lane3: CSFV purified virus control (C).

3.4.2 Development of the protocol for Indirect ELISA

The calculated OD values were plotted on Y-axis against different serum dilutions in X-axis. The point where the sample line cuts the PNT line is taken as a titer of the sample. The PNT baseline with different positive serum samples is presented diagrammatically and single serum dilution was calculated by linear regression (Figure: 3.3). The checker board titration of purified recombinant E2 protein showed 50 ng/well of protein being optimized for working range. The PNT curve for the calculation of the sample serum titer was obtained manually. ELISA using the single serum dilution method

was performed using 50 serum samples. The correlation coefficient of 0.9349 was calculated at 1:80 dilutions that were more than the calculated value for all other dilutions. Thus, 1:80 dilution of serum samples was further considered to predict the titer of remaining serum samples. The slope and intercept were 3.8246 and 0.0958, respectively. The observed and predicted OD for all the serum samples were calculated using the regression equation, Observed OD = 3.8246(predicted OD) +0.0958. A linear relation was observed between the predicted and observed titers (Figure: 3.3). The sensitivity, specificity, and accuracy of the assay relative to the commercial diagnostic ELISA are shown in Table.3.1. The sensitivity, specificity, and accuracy of recombinant E2 protein-based ELISA were calculated 97.65%, 92.50%, and 95.24%, respectively. Outline for methodology is also described in Figure: 3.1.

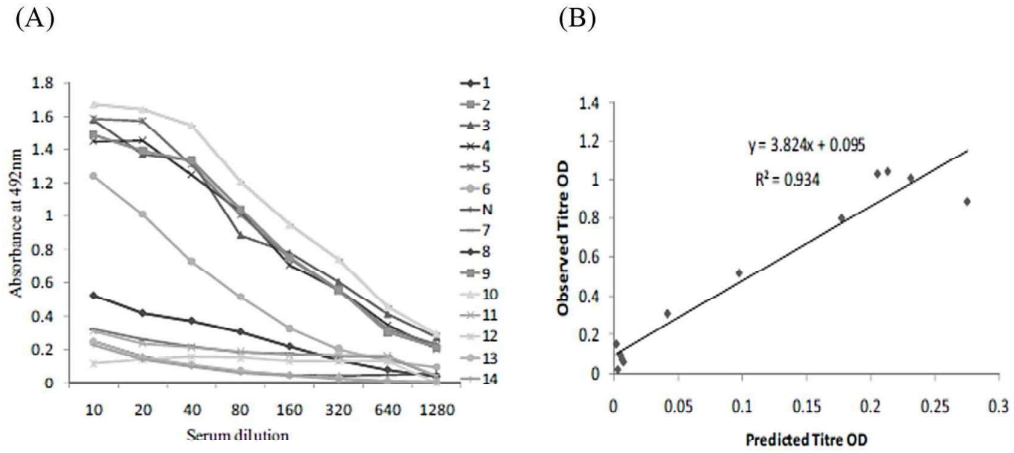

Figure: 3.3 The standard serial dilution method of determining observed CSFV ELISA antiserum titers from corrected absorbance with positive-negative threshold (PNT) baseline (A). N represents the PNT baseline while other lines show the different positive and negative serum samples. The relationship between the observed antibody titers of the serum samples obtained after serial dilution and their corresponding predicted antibody titers obtained from a single dilution ELISA at a 1:80 dilution (B).

	Commercial CSFV ELISA		
Single serum dilution ELISA	+ve	-ve	Total
+ve	166	3	169
-ve	4	37	41
Total	170	40	210

Table. 3.1 Relative sensitivity, specificity, and accuracy of the developed recombinant E2 protein-based ELISA to detect CSFV antibodies commercial CSFV ELISA assay as a reference standard.

Sensitivity: (166/170) × 100 = 97.65%.

Specificity: (37/40) × 100 = 92.50%.

Accuracy: (200/210) × 100 = 95.24%.

3.5 Discussion

CSFV shares antigenic epitopes with other pestiviruses such as border disease virus (BDV) and bovine viral diarrhea virus (BVDV) (Moennig 2000, Beer, Reimann et al. 2007). Although both BDV and BVDV are species-specific, they may infect pigs making the diagnosis of CSFV difficult in field condition (Lin et al., 2000). Eradication of CSFV from swine requires serological methods which are rapid and simple to perform. Moreover, the test should clearly differentiate between CSFV and other pestiviruses. In addition, the test should also detect antibodies early during the CSFV infection. In view of the above facts expression of CSFV specific E2 protein is necessary to differentiate CSFV from BDV and BVDV. In the present study, the E2 protein of CSFV was expressed from a bacterial expression system and the purified protein was used as an antigen for diagnostic ELISA. Recombinant protein-based serological tests are considered to have higher sensitivity and specificity as the target antigen is immuno-dominant and devoid of any non-specific moieties present in whole cell preparations. We have successfully cloned and expressed the E2 protein of CSFV using the bacterial expression system. Although the actual biological activity of E2 being a glycoprotein in the bacterially expressed form may not be justifiable in the absence of post-translational modification, its use in the diagnostics may have an added advantage. Our result showed a high yield of E2 protein (70 mg/l) from bacterial culture suggests its efficient production. Our study showed that E2 protein-based ELISA can be used for rapid and

efficient screening of a large number of serum samples, especially during the assessment of vaccination status of pigs involving large herd. The sensitivity and specificity of the E2 protein-based ELISA was 97.65% and 92.50%, respectively, relative to the commercial ELISA. The close correlation obtained between single serum and commercial ELISA titer showed similar trends in exposure to CSFV positive serum. However, the ELISA appears to be more sensitive than the commercial ELISA test and is able to detect antibody activity against CSFV in field samples that are not detectable by the commercial ELISA (Table 3.1). The ELISA reported in the paper using E2 protein would help to overcome some of the economic, technical, and statistical constraints of using this assay as a rapid serological assay against CSFV infection in pigs. The recombinant E2 protein-based single serum dilution ELISA for the detection of antibodies developed in this study was shown to be sensitive, specific, and accurate as compared to the available commercial ELISA. The study will help us to understand the use of viral surface protein in diagnostics of CSFV. These results demonstrate the potential benefit of a simple, specific ELISA for anti-E2 antibodies that may have diagnostic value for the pig industries. It will be interesting to explore the possibility of using other immunogenic proteins of CSFV for its diagnosis.

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Chapter IV

Production of rNDV expressing Erns protein of CSFV and functional analysis of its RNase activity

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Chapter IV

Production of rNDV expressing E^{rns} protein of CSFV and functional analysis of its RNase activity

4.1 Abstract

The structure protein E^{rns} of CSFV assists in the initial attachment of the virus to the host cell and its subsequent entry. 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 temperature of 50^0C. The study will be useful to express and characterize an enzymatic protein in its native form using the NDV as a vector.

4.2 Materials and methods

4.2.1 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 37^0C 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 (Hulst, van Gennip et al. 2000). 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 (Mittelholzer, Moser et al. 1998). The CSFV stock was stored at -80^0C 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 -80^0C.

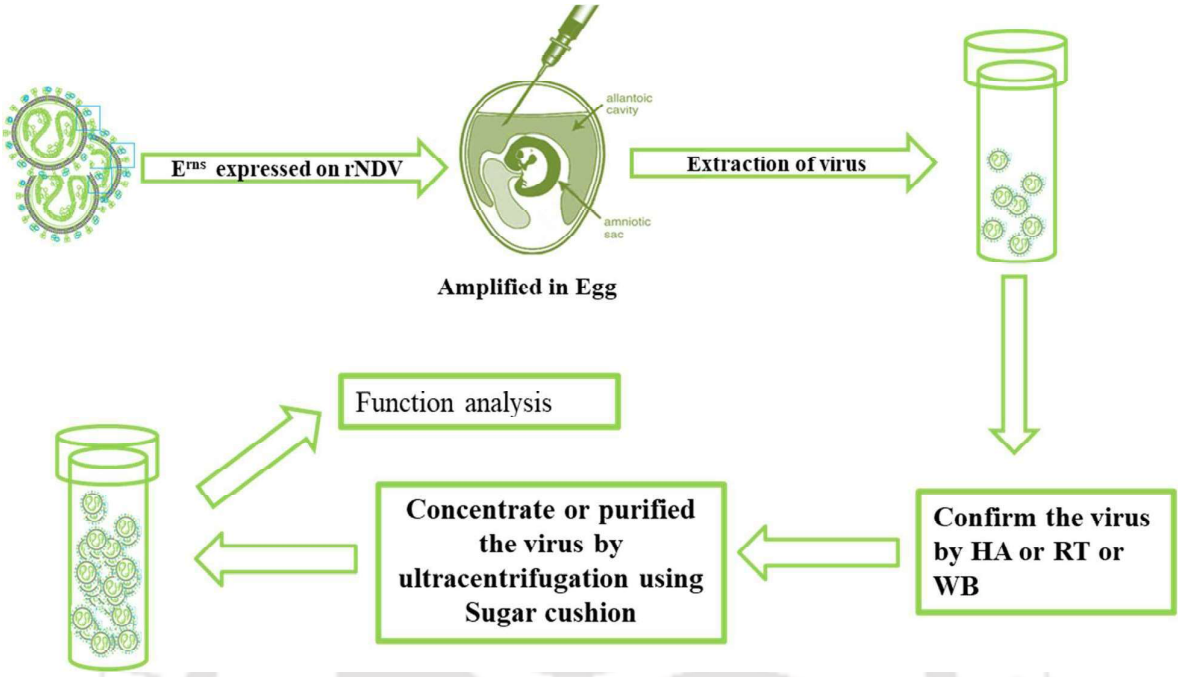

Figure: 4.1 Graphical representation of the used methodology

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

The NDV strain LaSota was used to construct antigenomic cDNA plasmid (Figure: 4.2). 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. The forward primer 5′A**GGCGCGCCT***TAAGAAAAAA***T***ACGGGTAGAA**GCCACC* **ATG**gaaaatataactcaatggaacct-3′ and reverse primer 5′-**AGGCGCGCCTTA**ggcataggcacc aaacca-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}.

4.2.3 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 (Alexander 2003).

4.2.4 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 *BamH*I and *Xho*I restriction sites. The integrity of the E^{rns} gene after cloning was confirmed by sequencing and restriction digestion. 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 His-tag antibody (Invitrogen, USA) and CSFV polyclonal antibody (obtained from IVRI, India).

4.2.5 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 XhoI 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).

4.2.6 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 NH4Cl 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 Mg2+ ions. Subsequently, Mg2+ was substituted by other metal ions like Mn2+, Ca2+, Ni2+, and Zn2+ 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 rErns. The effect of various salts (NaCl, KCl, and NH_4Cl) of different concentrations (50,100, 150 and 200 mM) on the RNase activity of rErns 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- HCl (pH 7.0–8.0), or CAPS (pH 9.0–10). The thermo-stability of rE^{rns} was determined by performing the reaction at a temperature range of 15–100 °C. The effect of divalent metal-ion on the RNase activity of rErns was determined by adding the 10 mM MgCl2 in the reaction mixture with and without of 10 mM EDTA (as a chelator).

4.2.7 Fluorescence titration of rE^{rns} specific RNAase activity

Fluorimetric titrations of the RNAase activity of rErns proteins was performed in a quartz cuvette containing 1 µM ethidium bromide (EtBr) solution (Jasco, Japan). The EtBr solution was titrated by the addition of different concentration of tRNA (Sigma–Aldrich) ranging from 5 µg to 40 µg. The reaction mixture was excited at 510 nm and emission was monitored from 520 to 700 nm using a 2.5/5 nm slit width in 25 mM Tris (pH 6.5) buffer containing 100 mM NaCl.

4.3 Results

4.3.1 Construction of pNDV-E^{rns} and recovery of rNDV-E^{rns}

The pNDV-E^{rns} showed the release of 720 bp E^{rns} gene of CSFV upon digestion with *Asc*I (Figure: 4.2B). Further digestion of pNDV-E^{rns} with *Apa*I showed the release of desired 1442 bp fragment (Figure: 4.2B). The nucleotide sequence of the complete pNDV-E^{rns} was found intact without any inadvertent mutation in the NDV genome and E^{rns} gene. The rNDV-E^{rns} was recovered by the established method using the support N, P, and L plasmids. The recovered rNDV-E^{rns} was found stable after 20 passages in the 9-day-old chicken embryo and PK-15 cells. The final passaged rNDV-E^{rns} showed amplification of the E^{rns} gene by RT-PCR and nucleotide sequencing. The 20th passaged rNDV-E^{rns} showed a titer of 2^8 (=256) HA unit and 2×10^8 PFU/ml in PK-15.

Furthermore, the rNDV-E^{rns} showed positive band of ~44 kDa corresponding to the E^{rns} protein of CSFV on western blot analysis (Figure: 4.2C).

4.3.2 Construction and expression of recombinant E^{rns} expressed in bacteria

The PCR amplified product of E^{rns} after RT-PCR was cloned into pET28a prokaryotic vector flanked with *BamH*I and *Xho*I. The restriction digestion showed the release of 681 bp cloned fragment after digestion with *BamH*I and *Xho*I (Figure: 4.3). Furthermore, the rE^{rns} protein gene was found intact after its nucleotide sequencing. The rE^{rns} expressed in BL21 cells as a fusion protein with His tag produced a ~35 kDa protein (Figure: 4.3). The average yield of the different fraction of the rE^{rns} protein was 0.8 mg/ml. Finally, purified fraction of the rE^{rns} protein showed a band of ~35 kDa on western blot (Figure: 4.3).

4.3.3 RNase activity assessment of rNDV-E^{rns}

The isolated cellular RNA was incubated with rNDV-E^{rns} in order to investigate its enzymatic activity. The rNDV-Erns showed complete degradation of the cellular RNA while rNDV as a negative control did not show any effect (Figure: 4.4). Furthermore, rNDV-E^{rns} showed complete degradation of the cellular RNA 1 h post incubation (Figure: 4.4B). The RNase activity of rNDV-E^{rns} was slightly higher in the presence of KCl than in NH_4Cl and NaCl (Figure: 4.4C). Optimum RNase activity of the rNDV-E^{rns} was observed at 150 and 200 mM concentration of KCl. Maximal RNase activity of rNDV-E^{rns} was observed at pH 5–6 (Figure: 4.4D). The optimum temperature of rNDV-E^{rns} specific RNase activity was calculated at the temperature of 45–50 °C (Figure: 4.4E). Thermo-stability of rNDV-E^{rns} suggested the loss of its RNase activity beyond 80°C.

4.3.4 Fluorimetric titrations of the E^{rns}

The change of fluorescence intensity at a given EtBr concentration with increasing concentration of RNA gave a hyperbolic graph (Figure: 4.5). The maximum fluorescence intensity of the EtBr after binding with RNA was observed at 581 nm. The degradation of RNA was monitored with the change in the fluorescence intensity of emission. The rE^{rns} in 1400 nM concentration showed 50% reduction in RNA.

4.3.5 RNase activity assessment of prokaryotic expressed E^{rns}

The isolated cellular RNA was incubated with rE^{rns} protein to observe its enzymatic activity. The rE2 protein of CSFV and RNaseA (NEB, USA) was used as a negative and positive control, respectively. The rE^{rns} and RNaseA showed degradation of the cellular RNA while rE2 did not show any effect (Figure: 4.6A). Furthermore, the rE^{rns} protein showed its maximum RNase activity at 2 h post incubation (Figure: 4.6B). The RNase activity of rE^{rns} was found higher in the presence of KCl than in NH4Cl and NaCl (Figure: 4.6C). In addition, NaCl at a concentration of 150 and 200 mM inhibits the RNase activity of the rE^{rns} protein. The rE^{rns} protein exhibited RNase activity from pH 4–10 (Figure: 4.6D). However, maximum activity was observed at pH 4–5 and decreased gradually beyond pH 5. Maximum RNase activity of the rE^{rns} protein was recorded at 45–50 °C (Figure: 4.6E). However, the rE^{rns} protein did not show any effect during incubation with RNA above 90 °C (Figure: 4.6F). The RNase activity of the rE^{rns} protein was inhibited by divalent metal ions. Zn2+ ions was found to be the most potent inhibitor of its RNase activity (Figure: 4.6G). Moreover, chelating metal ions (Mg2+), restores the RNase activity of the rE^{rns}. On the other side, Mg2+ ion was found to be the weakest inhibitors among all (Figure: 4.6 H).

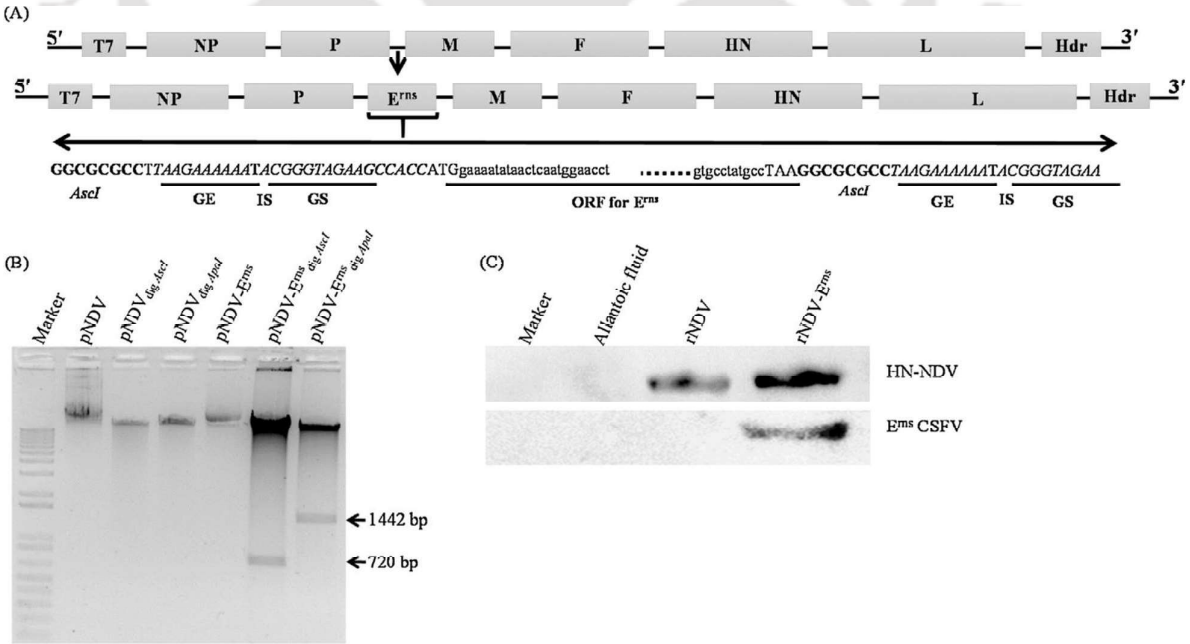

Figure: 4.2 Construction of recombinant plasmid pNDV-E^{rns} . Schematic representation of upper sense strand DNA of pNDV (T7 promoter, NP- nucleoprotein, P- phosphoprotein, M- matrix protein, F-fusion glycoprotein, HN- hemagglutinin-neuraminidase protein, L-large polymerase protein, and Hdr- hepatitis delta ribozyme) and lower sense strand DNA of pNDV-E^{rns}.

Transcription cassette encoding E^{rns} was cloned into the *Asc*I site between the P and M genes of the NDV antigenomic cDNA. The E^{rns} ORF has flanking sequence of NDV gene end (GE), an intergenic T nucleotide, and a gene start (GS) (A). $pNDV_{dig\mathit{AscI}}$ and $pNDV\text{-}E^{rns}{}_{dig\mathit{AscI}}$ represent the digestion of pNDV and pNDV-E^{rns} by *Asc*I to confirm the unique site of *AscI* and release of 720 bp (B). In addition, $pNDV_{dig\mathit{ApaI}}$ and showed release desired fragment confirming the orientation of cloned gene of the E^{rns} in pNDV-E^{rns} (B). Confirmation of NDV and E^{rns} specific protein expression by rNDV-E^{rns} by western blot using monoclonal antibody of HN and polyclonal antibody of CSFV, respectively (C).

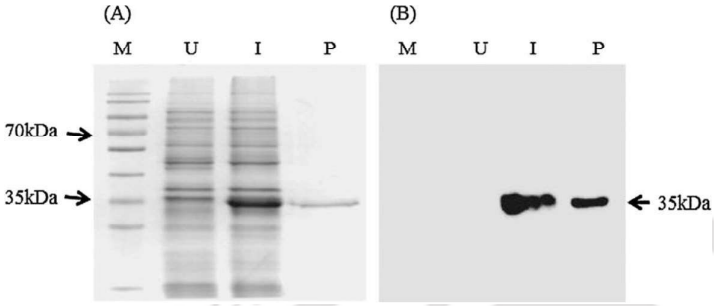

Figure: 4.3 Expression analysis of rE^{rns} protein in bacteria. The SDS-PAGE analysis of the E^{rns} protein of CSFV expressed in pET28a prokaryotic expression vector (A). M: protein molecular weight marker; U: uninduced recombinant pET28a containing E^{rns} protein gene; I: expression of protein in recombinant pET28a containing E^{rns} protein gene after 3 h of 0.5 mM IPTG induction; P: purified recombinant E^{rns} protein. Western blot analysis showing the presence of rE^{rns} protein using anti-His antibody (B).

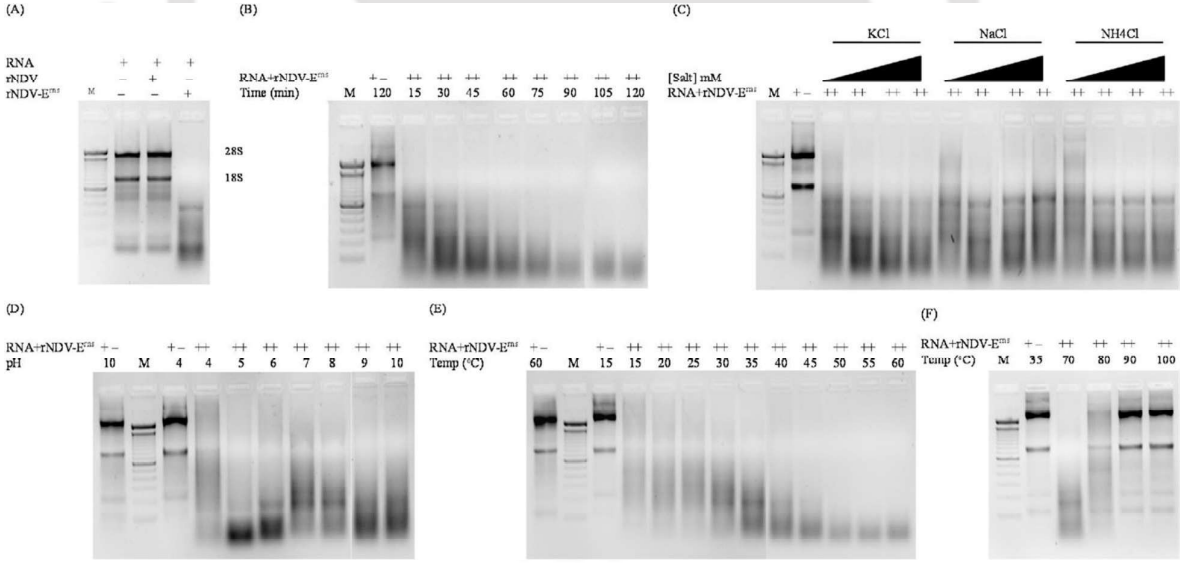

Figure: 4.4 Assessment of rNDV-E^{rns} enzymatic activity. RNase activity of rNDV-E^{rns} was specific to E^{rns} (A). Agarose gel showing RNase activity of rNDV-E^{rns} at the different time interval (B). Furthermore, agarose gel showing RNase activity of rNDV-E^{rns} in presence of various salts under concentration of 50 -200 mM (C) and different pH conditions (D). Agarose gel showing impaired enzymatic activity of rNDV-E^{rns} above 80 °C (F).

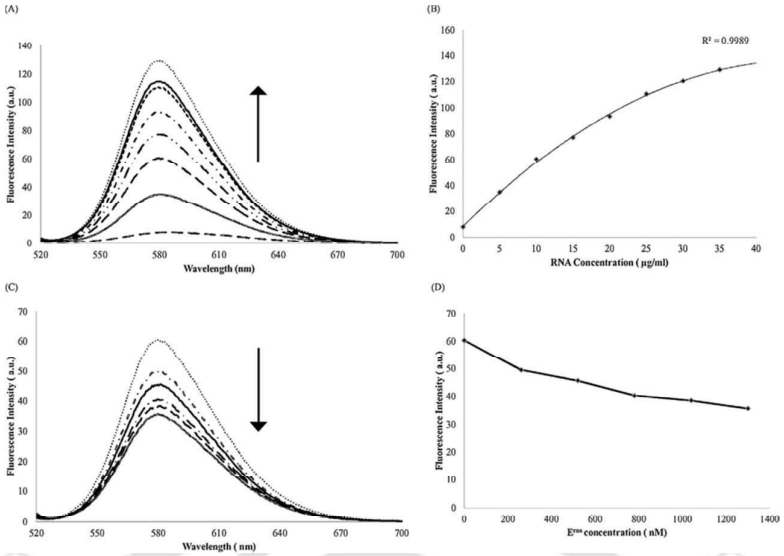

Figure: 4.5 RNase activity of rE^{rns} protein by spectrofluorimeter. Fluorescence spectra of EtBr (1 µM) in the presence of RNA (0–35 µg/ml); the arrow indicates increasing concentrations of RNA (A). Fluorescence intensity of EtBr (1 µM) in the presence of varying concentrations of RNA on excitation at 510 nm and emission at 581 nm (B). Fluorescence spectra of EtBr–RNA (1 µM) in the presence of rE^{rns} protein (0–1300 nM); the arrow indicates increasing concentrations of RNA (C). The relationship between the changes in fluorescence intensity of the EtBr–RNA system with increasing concentrations of rE^{rns} protein (0 to1300 nM) (D).

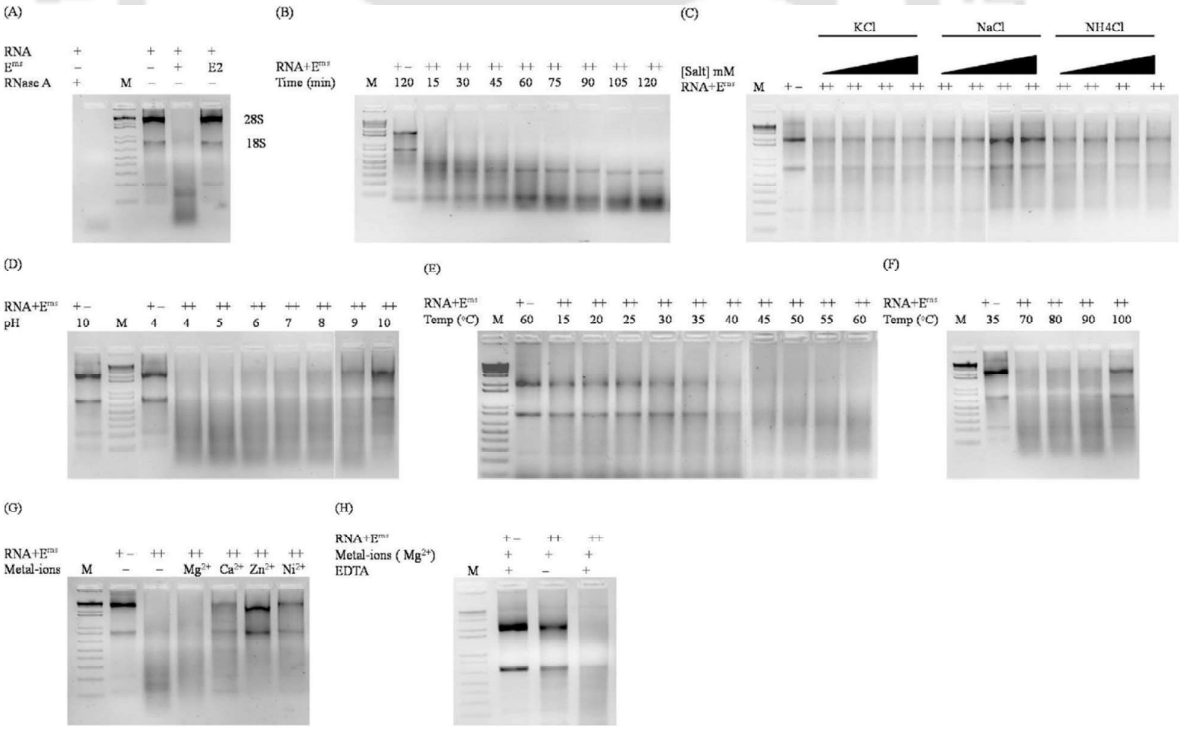

Figure: 4.6 Enzymatic activity assessment of rE^{rns} protein. RNase activity is specific to rE^{rns} protein and not by any other bacterial protein (A). Agarose gel showing RNase activity at the different time interval (B), in presence of various salts under concentration of 50–200 mM (C), and at different pH (D). Agarose gel showing RNase activity of bacterial expressed rE^{rns} at

different temperature (E). Effect of pre-heating treatment of rE^{rns} showing its impairment above 90 °C (F). Effect of divalent metal ions (G) and its chelation (H) on RNase activity of rE^{rns}.

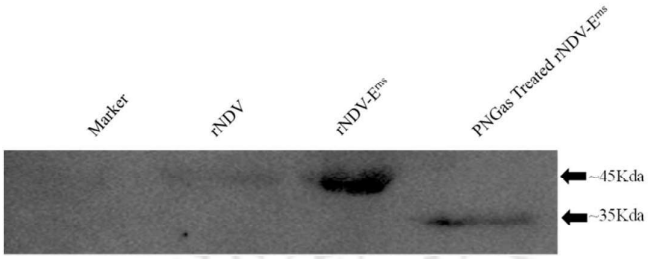

Figure: 4.7 Western blot of PNGase treated rNDV-E^{rns}. PNGase treated rNDV-E^{rns} showing deglycosylated 35Kda protein band.

4.4 Discussion

CSFV is an important pathogen of swine and its biology has been explored in order to make its proper diagnostics and vaccine (Munoz-Gonzalez, Perez-Simo et al. 2015, Kumar, Barman et al. 2016). Although RNase activity of the E^{rns} protein of CSFV has been discussed using yeast expression system to a certain extent, its activity using a mammalian host system has not been explored (Luo, Li et al. 2015). Our study of rNDV expressing E^{rns} protein of CSFV would be an ideal way to express its native form. Moreover, the E^{rns} expressed as part of the rNDV structural protein allowed to retain its folding by virtue of its stoichiometry. It has been shown earlier that protein expressed as a part of NDV will have high functionality (Swayne, Suarez et al. 2003, An, Liu et al. 2016). Presumably, rNDV-E^{rns} will have native conformation and glycosylation (Figure: 4.7). It is well established that inappropriate glycosylation can impact the enzymatic activity of a protein (Roberts 1960, Goettig 2016). The rNDV-E^{rns} showed an RNase activity with the cellular RNA suggesting the functional activity of the expressed recombinant protein. The rNDV-E^{rns} showed its optimum RNase activity at a temperature range of 45–50 °C and pH range of 4–7 consensus with the earlier report (Schneider, Unger et al. 1993, Windisch, Schneider et al. 1996).

To further strengthen the findings, we expressed the rE^{rns} in a prokaryotic expression system. In contrary to the earlier report, our study of prokaryotic expressed rE^{rns} showed RNase activity with the cellular RNA (Chen, Xia et al. 2007). The pattern of the protein folding largely depends on the condition of its expression and the accessory tag used (Costa, Almeida et al. 2014). The difference in our results might be due to the

expression vector used for the protein production and its folding, which may have exposed its functional domain. Furthermore, the rE^{rns} showed positive fluorimetric results suggesting the functional protein. It has been shown that RNA binding with EtBr could be used to assess the RNase activity (Tripathy, Dinda et al. 2013) Our results showed that fluorescence intensity of RNA bound EtBr decreased with increasing concentration of rE^{rns} suggested RNA degradation in presence of rE^{rns}. Moreover, the enzymatic activity of the E^{rns} expressed as a viral recombinant protein was quite higher than the one expressed by bacteria. We believe that expression of rNDV-rE^{rns} will have enhanced activity because of its native and glycosylated forms, which was absent in its bacterial form. However, the thermal stability of the rNDVrE^{rns} was lower as compared to a bacterial expressed form of rE^{rns}. Our results suggest that the stability of the rE^{rns} activity could be modulated by its glycosylation. It has been proposed that metal ions could modulate the RNase activity of the recombinant protein (Li, Chelladurai et al. 1993). In our study, the metal ion showed minimal effect on the RNase activity of the rE^{rns} corroborating to the earlier findings. However, a higher concentration of the Mg ions (> 10 mM) showed inhibitory effect on the RNase activity of the rE^{rns}. It has been shown that the RNase activity of E^{rns} leads to immune suppression of the host by inducing apoptosis of lymphocytes (Bruschke, Hulst et al. 1997). The E^{rns} protein of CSFV has been explored for its diagnostic potential (Pannhorst, Frohlich et al. 2015, Meyer, Fritsche et al. 2017). It will be interesting to explore the immunobiology of CSFV using rNDV-E^{rns}. The rNDV-E^{rns} will express the recombinant protein through normal infection in pigs. Moreover, infection of rNDV-E^{rns} will give us a better insight regarding its biology as compared to other expressed forms. The proposed methodology will be an ideal way of producing a highly soluble recombinant protein with its native conformation and function. Moreover, a single embryonated chicken egg will be able to produce high amount (~3 mg) of recombinant protein without involving any instrument. In addition, the methodology will be an ideal, rapid, and highly cost-effective way of producing recombinant enzymatic protein. The lentogenic strains of NDV have been routinely used for vaccine production and will be an ideal vector to produce recombinant proteins or enzymes.

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Chapter V

Evaluation of recombinant Newcastle disease virus expressing surface glycoproteins of classical swine fever virus: A vaccine candidate and an effective diagnostic for pig

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Chapter V

Evaluation of recombinant Newcastle disease virus expressing surface glycoproteins of classical swine fever virus: A vaccine candidate and an effective diagnostic for pig

5.1 Abstract

In the present study, E2 and E^{rns} glycoproteins of CSFV are expressed using rNDV platform. The rescued rNDV expressing E2 and E^{rns} proteins showed effective CSFV neutralization antibody titer upon intranasal vaccination in pigs. In addition, the vaccinated serum samples showed neutralization of both homologous and heterologous CSFV strains. Furthermore, the rNDV comprising E2 and E^{rns} proteins of CSFV were used to develop an indirect ELISA. This proposed methodology showed an insight that the E2 protein-based ELISA is better as compared to E^{rns}. Furthermore, the addition of E^{rns} along with E2 protein could reduce the efficacy of E2 based ELISA. The proposed methodology showed a possible vaccine strategy and an effective diagnostics for CSFV infection. This could be an economical and better alternative for existing vaccine and diagnostic for CSFV detection and control, respectively, in pigs.

5.2 Materials and Methods

5.2.1 Viruses and cells

Porcine kidney cell line (PK-15) and chicken fibroblast cells (DF-1) [ATCC, USA] were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were maintained in DMEM with 5% FBS. The vaccine (C-strain) and virulent (Assam) strains of CSFV were procured from College of Veterinary Sciences, Guwahati, India. The NDV strain LaSota was grown in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The recombinant vaccinia virus strain Ankara (a kind gift from Dr. Bernard Moss, NIH USA) was used to express T7 RNA polymerase for recovery of recombinant viruses. The stock of vaccinia virus was prepared in primary chicken embryo fibroblast cells using standard protocol (Kumar, Nayak et al. 2011).

5.2.2 Construction and recovery of rNDV expressing E2 and E^{rns} protein genes of CSFV

The NDV strain LaSota was used to construct antigenomic cDNA plasmid (GenBank accession number AF077761). A linker was designed which comprises six corresponding restriction sites present in NDV genome. The linker was designed to flank a T7 promoter and the Hdr sequence with T7 termination sequence, inserted between *Fse*II and *Pst*I restriction site in pUC19 vector (Figure: 5.1). The complete antigenome of NDV was divided into five fragments and cloned sequentially into the linker. A unique restriction site *Asc*I (3236nt to 3243nt) has been created between noncoding sequences of P and M gene of NDV by site-directed mutagenesis (Figure: 5.1). The total RNA was isolated from PK-15 cells infected with the vaccine strain of CSFV (C-strain) at 72 hrs post infection. The cDNA was synthesized using CSFV gene-specific reverse primer followed by its PCR. The amplified PCR product was gel purified and cloned into the *Asc*I site into the rNDV backbone. The complete E2 (2441-3559 corresponding to CSFV genome sequence) and E^{rns} (1175-1855 corresponding to CSFV genome sequence) glycoprotein genes of CSFV with gene-start and gene-end were cloned in full-length antigenomic cDNA of NDV (Figure: 5.1). The integrity and orientation of the E2 and E^{rns} genes were confirmed by restriction digestion and its nucleotide sequence analysis. The full-length NDV plasmid bearing the E2 and E^{rns} genes with gene-start and gene-end sequences of NDV was named as pNDV-E2csfv and pNDV-E^{rns}csfv. The recombinant virus was recovered following transfection of pNDV-E2csfv and pNDV-E^{rns}csfv in PK-15 cells with three accessory plasmids bearing the N, P and L genes of NDV as described earlier (Peeters, de Leeuw et al. 1999). The rescued recombinant viruses from above plasmids were named rNDV-E2csfv and rNDV-E^{rns}csfv. Both the rNDVs were plaque purified and amplified in 9-day-old embryonated SPF chicken eggs.

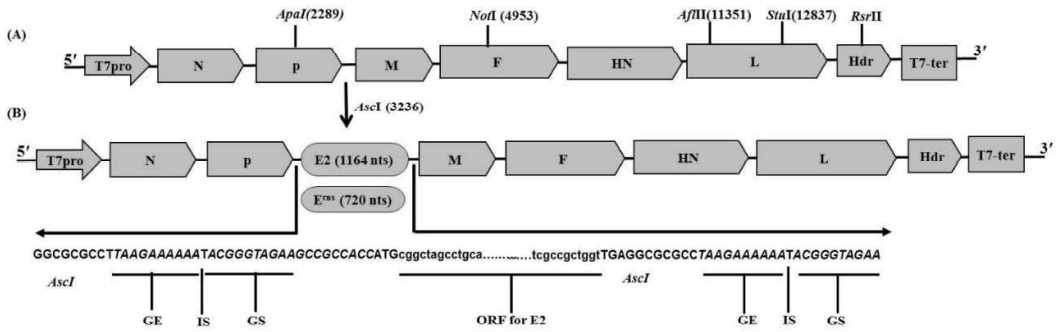


Figure: 5.1 Construction of the recombinant full length genome plasmid of Newcastle disease virus (NDV) (A). The sense strand DNA of pNDV contains T7 promoter followed by complete NDV genome and terminates with hepatitis delta ribozyme sequence. A unique restriction site *Asc*I was created at position 3236 corresponding to the full length pNDV. The *Asc*I site was used to clone the E2 and E^{rns} genes of classical swine fever virus (B). Transcription cassette (encoding E2 and E^{rns} genes) was cloned into the *AscI* site between the P and M genes of the NDV antigenomic cDNA. The GE, IS and GS corresponds to NDV gene-end, intergenic and a gene-start sequence, respectively.

5.2.3 Expression analysis of the recombinant NDVs

The E2 and E^{rns} protein gene expression by rNDV-E2csfv and rNDV-E^{rns}csfv, respectively, were confirmed by RT-PCR from isolated genomic RNA. Furthermore, the expression of the recombinant protein E2 and E^{rns} by rNDV was confirmed by western blot analysis using polyclonal CSFV antibody. The glycosylation pattern of the recombinant protein expressed by rNDV was checked by its digestion with PNGase (NEB, USA). The rNDV-E2csfv and rNDV-E^{rns}csfv were treated with PNGase at 37^0C for 30 mins and analyzed by immunoblotting.

5.2.4 Pathogenicity test and growth kinetics of the recovered rNDVs

Pathogenicity index test of the recombinant rNDV-E2csfv and rNDV-E^{rns}csfv was performed following the standard protocol (OIE 2012). The mean death time (MDT) was calculated after inoculating the rNDVs in 9-day-old embryonated SPF chicken eggs. The intracerebral pathogenicity index (ICPI) was performed in 1-day-old SPF chicks. Furthermore, the multistep growth kinetics was performed by infecting the recovered rNDVs in DF-1 cells at a MOI of 0.01 and collecting the supernatant every 8 up to 72 hrs post infection. The titers of rNDV-E2csfv and rNDV-E^{rns}csfv in the samples were quantified by 50% tissue culture infectious dose ($TCID_{50}$) assay in DF-1 cells. The allantoic fluid was added in the cell culture experiment as a source of external proteases.

5.2.5 Immunization study

Fifteen 120-days old pigs were randomly divided into five groups. The groups included three pigs each for rNDV-E2csfv, rNDV-E^{rns}csfv, rNDV and CSFV vaccination. Three animals were kept as unvaccinated controls. The pigs were housed in the separate pens as per their groups. All pigs were clinically healthy and maintained in the animal facility at the College of Veterinary Sciences Khanapra, Guwahati, India under standard conditions prescribed by the institutional guidelines. The vaccination protocol for the pigs was approved by the Institutional Animal Care and Use Committee. All the animals were

tested negative for CSFV specific antibody using commercial CSFV detection kit (IDEXX, Switzerland) and a TaqMan real-time RT-PCR. The pigs in group rNDV-E2csfv, rNDV-E^{rns}csfv and rNDV were vaccinated intranasally by 10^3 $TCID_{50}$/ml of the virus. Similarly, CSFV vaccines (C-strain) were inoculated to the control pigs as per the prescribed dose of 10^3 $TCID_{50}$/ml by intramuscular route. The animals were observed daily and temperatures were recorded. In addition, serum samples were collected on days 0, 1, 3, 5, 7, 14, and 21 post-vaccination. The boosters of the vaccines were given on day 21st post immunization and serum samples were collected on days 28th and 35th. The collected serum samples were checked for NDV replication by hemagglutination inhibition (HI) assay. On the other side, the serum samples were checked for virus neutralization using CSFV. The 10^3 $TCID_{50}$ CSFV per well were neutralized from collected serum samples following its four-fold dilution. The neutralizing antibody titers specific to CSFV were expressed as the reciprocal of the highest serum dilution that could inhibit the infection of PK-15 cells in 50% of the culture wells. The results of the neutralization test were validated using positive and negative reference sera. The neutralization ability of the serum samples was also checked for a heterologous virulent CSFV strain (Assam) isolated from an outbreak in India (Khatoon, Barman et al. 2017).

5.2.6 Development of ELISA assay using rNDVs

A total of 503 random pig serum samples were procured from ICAR Research Complex Meghalaya, Shillong and Veterinary College, Guwahati, Assam. The rNDV-E2csfv and rNDV-E^{rns}csfv were purified using 30% sucrose cushion at 35000 rpm for 2 hrs. The purified rNDV-E2csfv and rNDV-E^{rns}csfv were dissolved in carbonate and bicarbonate buffer (pH ~9.6) and coated onto a 96-well flat bottom polysorb plate (Nunc, USA) and incubated at 4^0C overnight. The protocol was used for rNDV-E2csfv and rNDV-E^{rns}csfv alone and in combination. The concentration of protein per well was determined by the checkerboard titration method as described earlier (Robinson et al., 1985). The wells were coated with 50µl (10^5 $TCID_{50}$/ml) of rNDV-E2csfv and rNDV-E^{rns}csfv alone and in combination. The coated wells were washed with phosphate buffer saline containing Tween-20 (PBST) and blocked by 5% lactalbumin hydrolysate for 1 h at 37^0C. All collected serum samples were diluted to 10^3 times to reduce the higher saturated reading. The normalized serum samples were diluted serially (till endpoint) in the recombinant virus coated plates and incubated for 1 h at 37^0C. The wells were washed with PBST and further incubated with 50µl (1:10^4 dilution) of the HRP-conjugated anti-

pig antibody (Pierce, USA) for 1h at 37^0C temperature. The plates were washed with PBST and serum samples were detected with 50µl of TMB (Invitrogen, USA) after 15 min incubation at 37^0C temperature. The enzymatic reaction was stopped by adding 50 µl of 1M H_2SO_4, and absorbance was recorded at 450 nm in a plate reader (Thermo Scientific, USA).

A line plotted from positive serum samples from different pigs were used for the upper baseline antibody titer. Similarly, a line was plotted from the negative samples to define the lower baseline titer. The negative serum samples were used to construct positive-negative threshold (PNT) baseline as shown earlier (Snyder, Marquardt et al. 1984, Kumar, Barman et al. 2016). Each positive and negative serum was analyzed in three wells to plot the positive and PNT curve, respectively. The maximum dilution that crosses the PNT line is called observed OD or dilution. This can be considered as an observed titer of the sample. Any serum sample showing an OD above PNT line on selected dilution was considered positive. Similarly, the mean OD of each serial dilution of many hyperimmunized positive sera was taken to plot the curve. The respective OD of respective dilution of many sera gave the average OD on each dilution. The sensitivity, specificity, and accuracy were calculated by standard mathematical derivations (Snyder, Marquardt et al. 1984). The serum samples were further validated by commercially available IDEXX kit for CSFV.

5.3 Results

5.3.1 Construction and recovery of rNDV expressing E2 and E^{rns} protein gene of CSFV

The pNDV was successfully constructed with unique restriction site *AscI* and its integrity was confirmed by sequencing. The insertion of E2 and E^{rns} genes in pNDV-E2csfv and pNDV-E^{rns}csfv was confirmed by digestion with *AscI and ApaI* enzymes and release of 1164bp and 720bp fragments, respectively (Figure: 5.2A). The clones were found intact upon nucleotide sequencing. The full-length antigenomic plasmids pNDV, pNDV-E2csfv, and pNDV-E^{rns}csfv transfected along with three accessory plasmids of N, P and L rescued the rNDV, rNDV-E2csfv, and rNDV-E^{rns}csfv, respectively. The RT-PCR analysis and their complete genome sequencing confirmed the genome stability of rescued rNDV, rNDV-E2csfv, and rNDV-E^{rns}csfv.

5.3.2 Characterization of the rNDVs expressing CSFV protein genes

The recovered rNDV-E2csfv and rNDV-E^{rns}csfv showed stable expression of the foreign gene for up to twenty passages in 9-days-old embryonated eggs and in DF-1 cells. The E2 and E^{rns} protein genes in rNDV-E2csfv and rNDV-E^{rns}csfv showed positive amplification on RT-PCR analysis and its nucleotide sequencing (data not shown). The incorporation of CSFV proteins into rNDV virion was confirmed by western blot analysis of the purified virion. The NDV HN specific monoclonal antibody (a kind gift from Dr. R Iorio, University of Massachusetts, Worcester, MA, USA) showed a band of ~74 kDa corresponding to HN protein in rNDV, rNDV-E2csfv, and rNDV-E^{rns}csfv (Figure: 5.2B). The CSFV polyclonal antibodies showed a band of 55 kDa (E2) and 44 kDa (E^{rns}) in rNDV-E2csfv and rNDV-E^{rns}csfv lanes, respectively (Figure: 5.2C). The PNGase treatment of rNDV-E2csfv and rNDV-E^{rns}csfv showed a reduction in respective band size (Figure: 5.2D). The rNDV-E2csfv and rNDV-E^{rns}csfv showed titers of ~ 2^8 HA unit from the infected allantoic fluid. The MDT and ICPI values of both the rNDV-E2csfv and rNDV-E^{rns}csfv were found >120 h and 0, respectively. The growth kinetics of all the rNDVs showed similar kinetics in DF-1 cells (Figure: 5.2E).

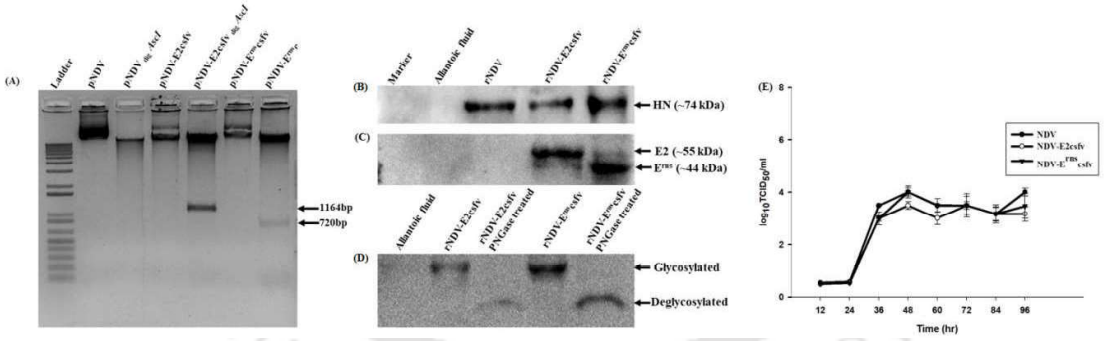


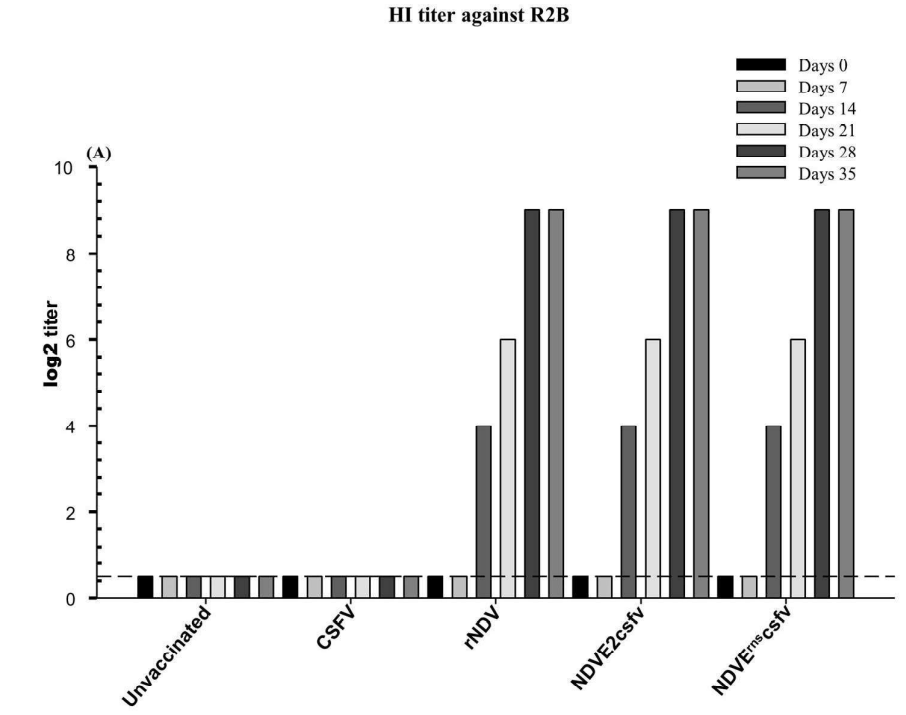
Figure: 5.2 Restriction map for the pNDV, pNDV-E2csfv and pNDV-E^{rns}csfv (A). Restriction digestion of pNDV, pNDV-E2csfv, and pNDV-E^{rns}csfv showing released fragments of 1164 bp and 720 bp of E2 gene and E^{rns} genes, respectively. The protein expression of the purified recombinant viruses, rNDV, rNDV-E2csfv, and rNDV-E^{rns}csfv by western blot analysis showing 74kDa NDV HN protein specific band (B). Similarly, the purified rNDV-E2csfv and rNDV-E^{rns}csfv showing 55 kDa and 44 kDa protein bands of E2 and E^{rns}, respectively using CSFV polyclonal antibodies (C). The western blot showing the glycosylated form of the CSFV glycoproteins on the treatment of purified rNDV-E2csfv and rNDV-E^{rns}csfv with PNGase (D). The growth kinetics of rNDV, rNDV-E2csfv and rNDV-E^{rns}csfv in DF-1 cells at different time points post-infection (E).

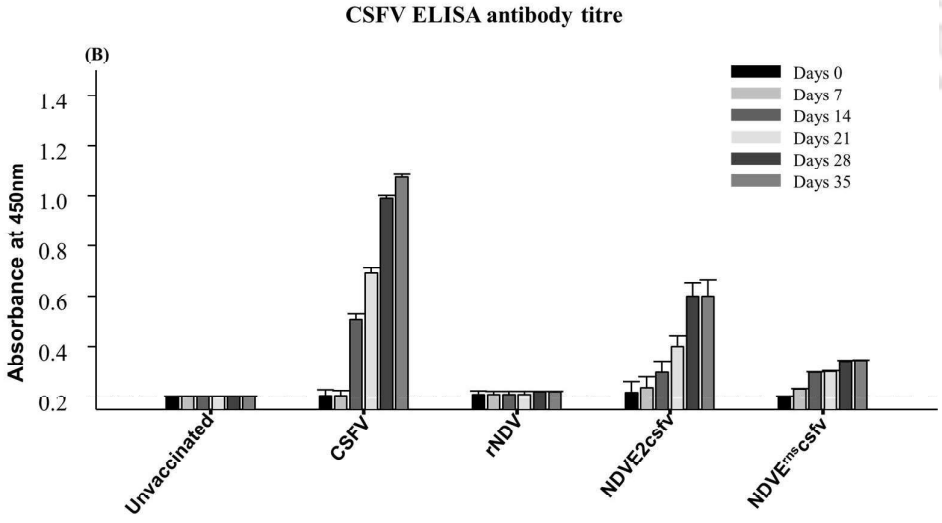
5.3.3 Immunization and seroconversion

No clinical signs and symptoms were observed in the pigs vaccinated with the rNDVs, rNDV-E2csfv, and rNDV-E^{rns}csfv. However, the CSFV vaccinated pigs showed slightly elevated temperature on day 3rd post vaccination. All the vaccinated pigs except control animals showed seroconversion against CSFV and NDV post vaccination. HI titer of 2^6 was recorded in rNDV, rNDV-E2csfv and rNDV-E^{rns}csfv vaccinated pig on day 21st. The titers were increased following a booster dose to 2^9 HI units in all the rNDVs vaccinated groups (Figure: 5.3A). No HI titer was recorded in control and CSFV vaccinated groups.

5.3.4 Serum antibody and CSFV neutralization titers

The maximum CSFV antibody titer was recorded in CSFV vaccinated group followed by NDV-E2csfv and then in NDV-E^{rns}csfv (Figure: 5.3B). The serum samples collected from rNDV-E2csfv and rNDV-E^{rns}csfv vaccinated pigs showed neutralization titers (log_2) of 2^8 and 2^6 against the vaccine strain of the CSFV (Figure: 5.3C). Another virulent strain of CSFV (isolates from an outbreak in India) was used to check for the cross-neutralization titer against its heterologous strains. The serum samples from rNDV-E2csfv and rNDV-E^{rns}csfv vaccinated pigs showed neutralization titers (log_2) of 2^7 and 2^5 against the virulent strain of CSFV (Figure: 5.3C). However, the CSFV vaccinated group showed the highest neutralization titer against both of its homologous and virulent strains. No neutralization against CSFV was recorded from serum collected from unvaccinated and rNDV control pigs.


HI titer against R2B
(A)
log2 titer
10
8
6
4
2
0
Days 0
Days 7
Days 14
Days 21
Days 28
Days 35
Unvaccinated
CSFV
rNDV
NDVE2csfv
NDVEmscsfv


CSFV ELISA antibody titre
(B)
Absorbance at 450nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Days 0
Days 7
Days 14
Days 21
Days 28
Days 35
Unvaccinated
CSFV
rNDV
NDVE2csfv
NDVEmscsfv

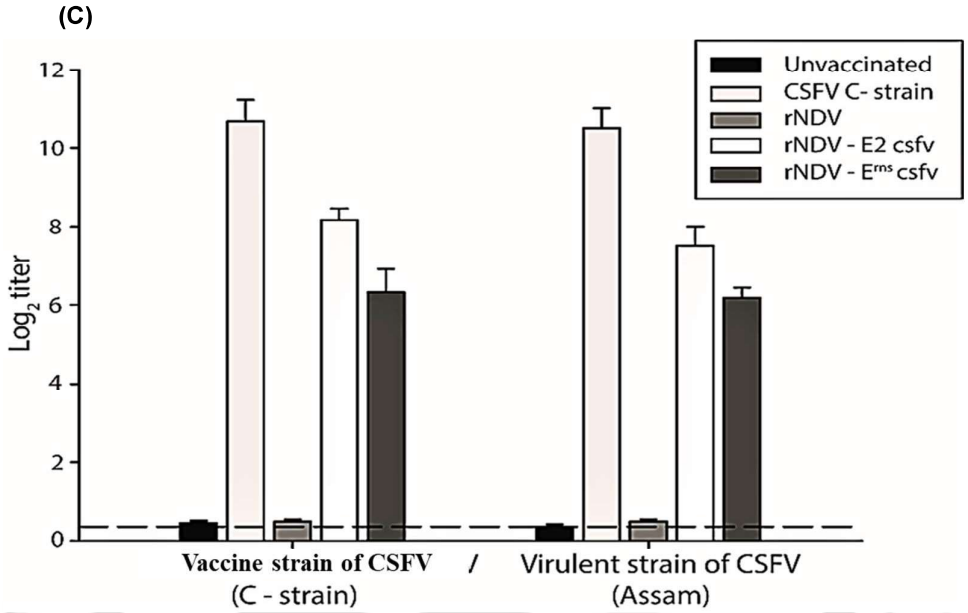


Figure: 5.3 Serology of the pigs vaccinated with recombinant NDV expressing surface proteins of CSFV. The graph showing the HI antibody titers of the serum samples collected from the pigs vaccinated with rNDV, rNDV-E2csfv and rNDV-E^{rns}csfv at different time intervals (A). A graph showing the CSFV antibody titers from all the group at different time intervals post vaccination (B). A bar graph showing the neutralization of two CSFV strains (C).

5.3.5 ELISA assay

The average of all OD values at each respective dilution was taken and +3σ (+3 standard deviation) above of all average points applied to plot PNT curve (Figure: 5.4). All the average absorbance values were plotted against their respective dilution. The dilution of 1:8000 acquired maximum regression coefficient after plotting the curve between observed OD/dilution and predicted OD/dilution. The rNDV-E2csfv coated ELISA showed the highest regression value of 0.896 followed by rNDV-E^{rns}csfv (Figure: 5.5). The least regression value of 0.868 was calculated for the combined ELISA (both rNDV-E2csfv and rNDV-E^{rns}csfv). Similar findings were recorded for the absorbance reading where rNDV-E2csfv showed the higher value as compared to rNDV-E^{rns}csfv alone and in combination (Figure: 5.6). A total of 503 serum samples from different pigs were used to calculate the sensitivity, specificity, and accuracy of the rNDVs based ELISA. The sensitivity, specificity, and accuracy of the rNDV-based ELISA were 96.34%, 96.88%, and 96.62%, respectively, relative to the available commercial CSFV ELISA kit (Table 5.1).

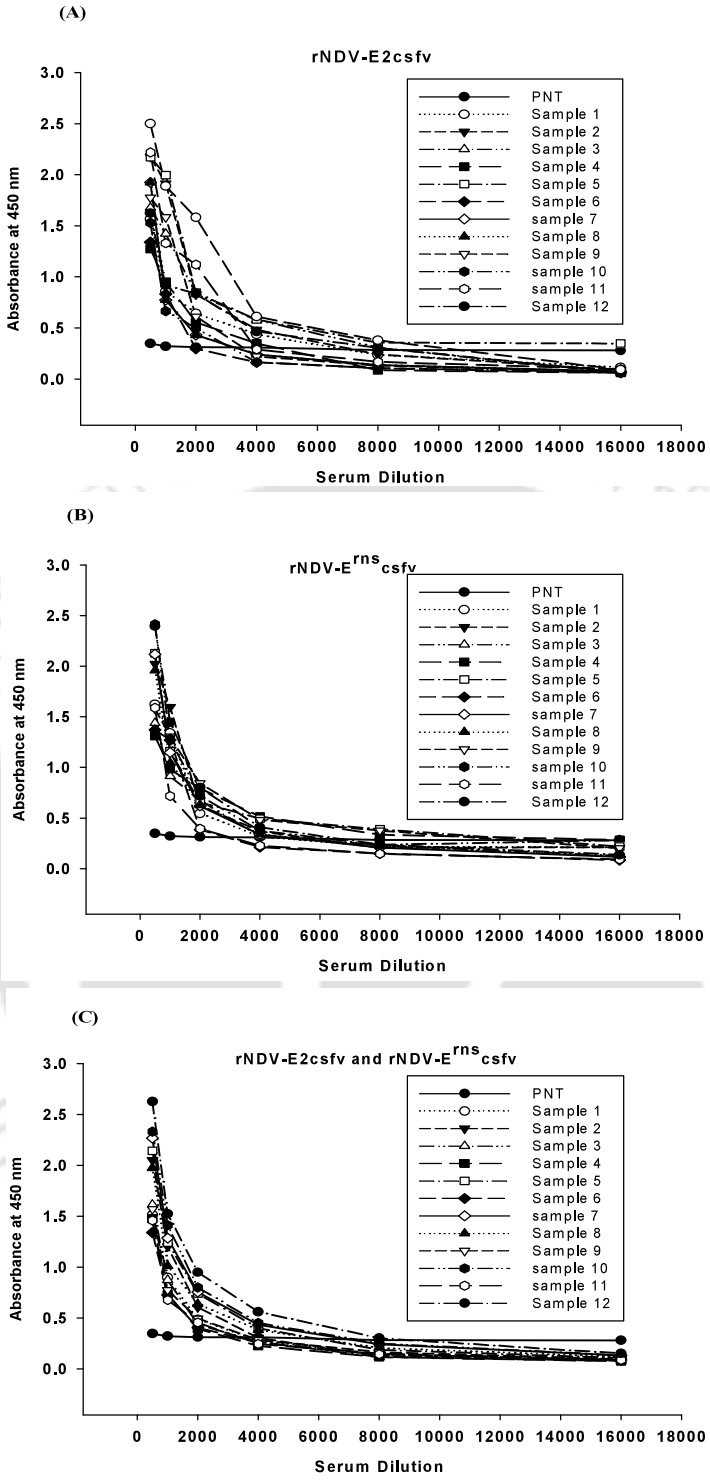


Figure: 5.4 The serial dilution of serum samples to determine the observed antibody titres for rNDV-E2csfv (A), rNDV-E^{rns} csfv (B) and in combination (C) against classical swine fever virus. The corrected absorbance of all the three rNDVs was plotted with the positive-negative threshold (PNT) baseline.

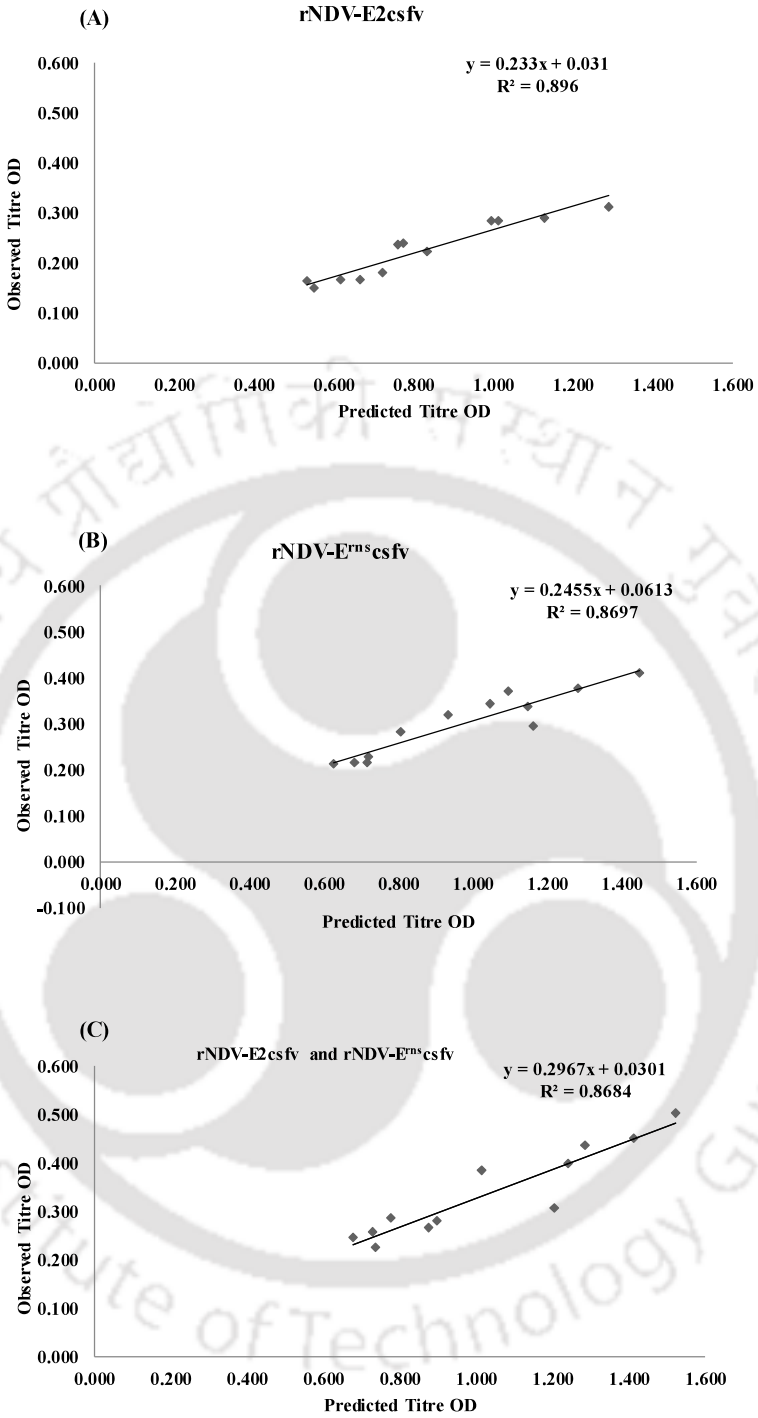


Figure: 5.5 The regression analysis of rNDV-E2csfv (A), rNDV-E^{rns}csfv (B) and their combined (C) ELISA. The regression analysis defines the relationship between the observed antibody titres of the samples recorded after serial dilution and their corresponding predicted antibody titres from a single dilution at 1:1000 dilution.

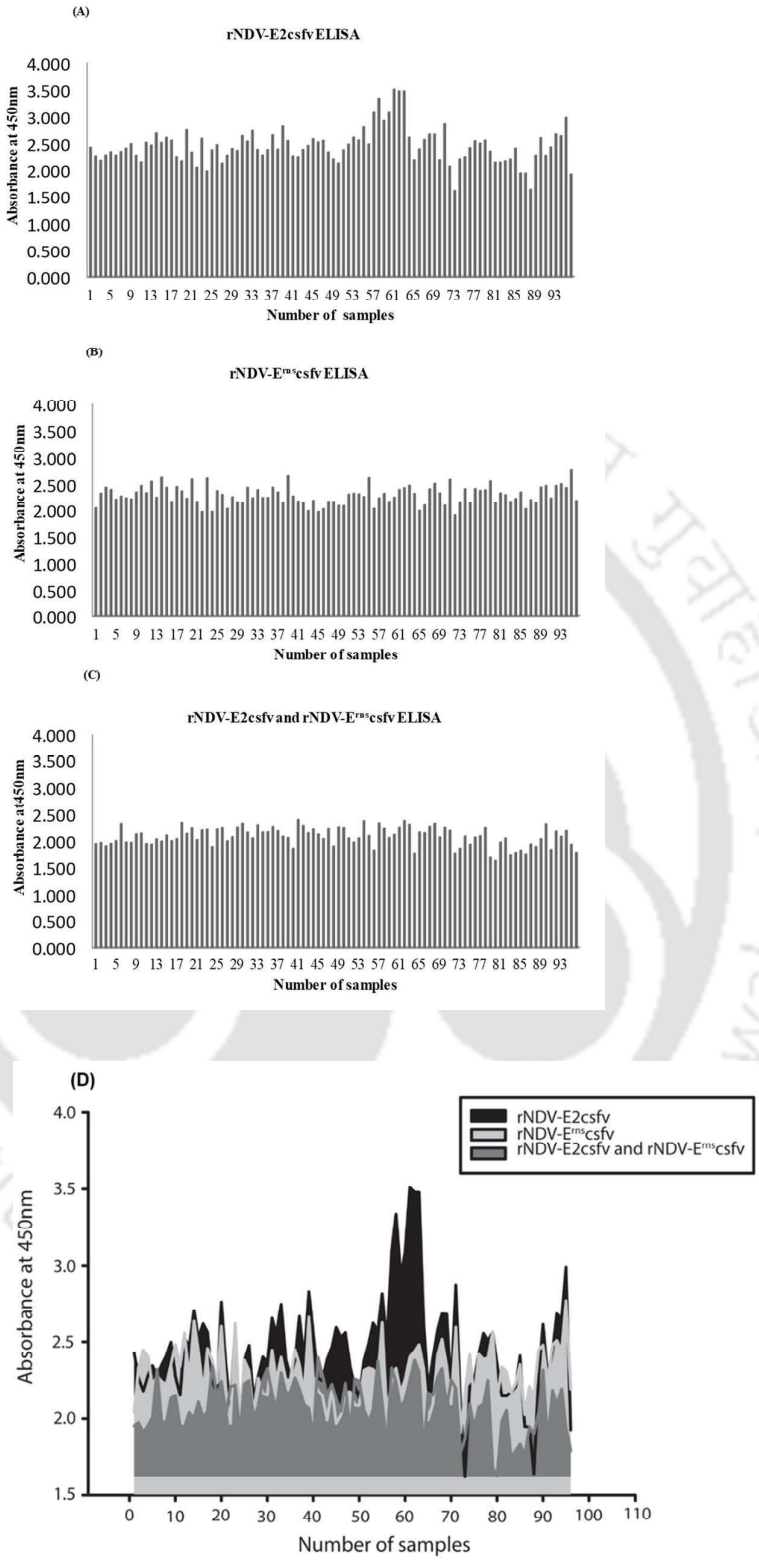


Figure: 5.6 The rNDV-E2csfv and rNDV-E^{rns}csfv were coated alone and in combination for detecting the absorbance of the CSFV serum sample. The absorbance value of the negative serum samples were subtracted in order to get the actual absorbance of the CSFV positive samples. The

rNDV-E2csfv ELISA is showing higher absorbance compared to rNDV-E^{rns}csfv alone and in its combination. A merge of all ELISA graph (D).

	Gold standard CSFV ELISA		
rNDV ELISA	+ve	-ve	Total
+ve	237	8	245
-ve	9	249	258
Total	246	257	503

Table 5.1 Relative sensitivity, specificity, and accuracy of the developed recombinant NDV based diagnostics to detect CSFV specific antibodies.

Sensitivity: (237/246) x 100 = 96.34%

Specificity: (249/257) x 100 = 96.88%

Accuracy: (486/503) x 100 = 96.62%

5.4 Discussion

The E2 is the most immunogenic protein of CSFV and has been used as a subunit vaccine against CSFV (Madera, Gong et al. 2016, Suarez, Sordo et al. 2017). In addition to E2, E^{rns} protein has also been shown to induce the neutralization antibody response (van Zijl, Wensvoort et al. 1991, Hulst, Westra et al. 1993, Konig, Lengsfeld et al. 1995, Kosmidou, Buttner et al. 1998, Bouma, De Smit et al. 2000, Zhang, Yu et al. 2006). Glycosylation of the surface protein is an important factor that plays an important role in the immunogenicity of many envelope viruses (Haigwood, Nara et al. 1992, Fournillier, Wychowski et al. 2001). In case of CSFV, bacterial expressed E1, E^{rns}, and E2 proteins were unable to produce virus-neutralizing antibody response and also protection against CSFV infection (Gavrilov, Rogers et al. 2011). Presumably, the glycosylated form of the proteins could be a possible alternative for the development of a vaccine and diagnostic against CSFV infection in pigs.

The rNDV based vaccine approach will be useful for the pigs. The rNDV expressing the surface protein of CSFV has not been reported. The embryonated chicken

egg used to generate the large quantity of rNDVs to vaccinate pig will be an efficient and economical way in terms of its production. In addition, rNDV expressing the surface proteins of CSFV showed its glycosylated and functional form suggesting it to be an ideal antigen. The rNDVs expressing the surface glycoproteins of CSFV was found to be attenuated in the embryonated eggs and chicks suggesting the incorporation of foreign protein have not increased its virulence and pathogenicity, respectively. Moreover, the growth kinetics of the rNDVs expressing the proteins of CSFV showed the similar kinetics in the DF-1 cells when compared to its wild-type suggesting no enhancement in its cytopathic effect.

Our results of intranasal inoculation and seroconversion against both NDV and its expressed protein suggested it to be a good viral vector for swine. The intranasal route of inoculation would be an ideal way to vaccinate pigs, which could exclude its handling. The HI antibody titer of the different vaccinated group suggested the active replication of the rNDV in pigs. Differentiation of infected from a vaccinated animal (DIVA) is a great requirement to control CSFV in field condition. Importantly, pigs infected with CSFV could be differentiated from a vaccinated by the use of rNDV vectors. Our rNDV expressing CSFV surface proteins will be an ideal DIVA strategy. The results of our complementary ELISA will be useful in order to differentiate the infected with a vaccinated pig. The rNDV-E2csfv showed better CSFV neutralization antibody titers as compared to rNDV-E^{rns}csfv suggesting E2 be a better antigen than E^{rns}. Our findings corroborate with the earlier report, which showed the reduction in antigenicity of the recombinant virus upon addition of multiple glycoproteins (Kumar, Nayak et al. 2011). Our result will be a step forward in using rNDV to generate the vaccine against important animal pathogens (Khattar, Collins et al. 2010, Kortekaas, Dekker et al. 2010, Zhao, Spatz et al. 2014, Zhao, Sun et al. 2017).

The major problem of CSFV detection includes lack of cytopathic effect and low replication ability *in vitro* (Wang, Lu et al. 2000). The PK-15 and SK-6 cells are recommended for its routine *in vitro* infectivity assay (OIE 2008). The infection of CSFV could be diagnosed by commercially available ELISA kits (Schroeder, von Rosen et al. 2012, Pannhorst, Frohlich et al. 2015). The rNDV expressing surface glycoproteins of CFSV based ELISA could be a reliable and effective way for detecting CSFV specific antibodies from the serum samples. The system and method proposed could be an efficient and cost-effective way to produce a large amount of native antigen. The rNDV

expressing the surface protein of CSFV will be an ideal way to produce its antigen. Presumably, rNDV expressed the recombinant proteins of CSFV in native conformation, allowing its detection by the antibody. The ease of its purification using a sucrose cushion is an added advantage. The recombinant protein expressed by the rNDV showed its glycosylation suggesting an immunogenic version of the protein. Similar to the vaccine studies, the results of ELISA using rNDV-E2csfv showed better absorbance as compared to rNDV-E2csfv, suggesting E2 to be a better antigen as compared to E^{rns}. Moreover, the addition of E^{rns} would probably reduce the detection threshold of the assay. Presumably, the addition of E^{rns} would dilute the antigen concentration leading to reduce the sensitivity of the E2. Our finding also highlighted the preference of E2 in place of E^{rns} for any vaccine and/or diagnostic formulation against CSFV in swine. High sensitivity and specificity of the developed ELISA suggests the robust detection of the CSFV specific antibodies from the pigs.

The rNDV expressing E^{rns} and E2 showed its immunogenicity in the pigs suggested its use as a vector to express choice of antigens for the development vaccine against other pig pathogens. Moreover, pigs vaccinated with rNDVs showed high virus neutralization titer suggesting its effective immunogenicity inside host tissues. We could not perform the challenge experiment because of animal ethical concerns. However, it will be interesting to see the rNDV expressing the CSFV immunogenic proteins for vaccine and its efficacy post challenge in the pig. From the present study, it could be inferred that rNDV could be an alternate way to generate a recombinant vaccine and a possible choice for producing diagnostic antigens against CSFV. The results will be useful for the researchers working in the vaccine and diagnostic aspects of CSFV. In addition, the methodology used in the present work should be applicable to the larger species like pig where higher vaccine doses are required.

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Indian Institute of Technology Guwahati

Future prospects

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Future prospects

The development of improved and cost-effective vaccines and diagnostic are required to control the CSFV infection in endemic area. The CSFV virion encodes four structural proteins, namely, C, E^{rns}, E1, and E2. Among these the E2 and E^{rns} are immunogenic proteins. The surface proteins of CSFV are major immunogenic proteins. The combination of the surface proteins could be an alternate to develop a better vaccine against CSFV. Alternatively, the chimeric or fusion protein containing the major surface protein could also be explored.

NDV as a vector has been explored for development of recombinant vaccine. Many foreign proteins are expressed using NDV. Our study showed the use of rNDV for vaccine and diagnostic against CSFV. The optimum expression of rNDV depends on various factors, including size and confirmation. The expression of only the epitopic domain of surface glycoproteins of CSFV could be an alternate may to design the rNDV vectors. It will be interesting to check the threshold dose of rNDV for pigs. The NDV generally infects through occulo-nasal route. The rNDV is an ideal candidate for designing vaccine against larger species such as pig. Alternatively, the vaccine could be tried using aerosol route to see its immunogenicity.

To enhance the immunogenicity, rNDV could be included with same pig cytokines such as IL-2 or IFNγ. The addition of cytokines in the vaccine formulation has been shown to increase the efficacy of vaccine candidates. Alternatively, an rNDV could be designed that express the pig IL-2 or IFNγ along with the E2 or E^{rns} proteins of CSFV.

Prime boost is an upcoming strategy to potentiate the vaccine efficacy. It would be interesting to use different genotypic viruses, which do not show any cross reactivity. Different non-cross reactive antigen could be used alternatively to prime and boost vaccination.

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Research Achievements

भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

Research Achievements

List of published papers and conferences

1. Evaluation of recombinant Newcastle disease virus expressing surface glycoproteins of classical swine fever virus: A vaccine candidate and an effective diagnostic for pig
   Rakesh Kumar, Vishnu Kumar, Puro Kekungu, Nagendra N Barman, Sachin Kumar
   (Communicated to Virology Journal)

2. Kumar, R., V. Kumar and S. Kumar (2018). "Production of recombinant Erns protein of classical swine fever virus and assessment of its enzymatic activity: A recombinant Newcastle disease virus-based approach." Process Biochemistry **66**: 113-119.

3. Kumar, R., N. N. Barman, E. Khatoon and S. Kumar (2016). "Development of single dilution immunoassay to detect E2 protein specific classical swine fever virus antibody." Vet Immunol Immunopathol **172**: 50-54.

4. Kumar, R., N. N. Barman, E. Khatoon, G. Rajbongshi, N. Deka, S. Morla and S. Kumar (2015). "Molecular characterization of E2 glycoprotein of classical swine fever virus: adaptation and propagation in porcine kidney cells." In Vitro Cell Dev Biol Anim **51**(5): 441-446.

National Conferences/Seminars

I. XXXI Annual Convention of IAVMI, 2018. Sri Venkateswara Veterinary University, Tirupati
   Title: Diagnostics and vaccine development of classical swine fever virus based on recombinant Newcastle disease viral vector.

II. Shortlisted for presentation at Assam Biotech Conclave, 2017 (Guwahati Biotech Park)
   Title: Rapid and Cost effective diagnostic for classical swine fever.

III. Society for Veterinary sciences and Biotechnology (2017)
   Title: Expression of recombinant Erns protein of classical swine fever virus and characterization of its enzymatic activity: A recombinant Newcastle disease virus based approach.

IV. Research Conclave 2017, IIT Guwahati, Guwahati, Assam
   Title: Development of Indirect ELISA for classical swine fever.

V. Symposium on Biology and Molecular Pathogenesis of Viruses, Indian Institute of Science, Bangalore (June, 2016)
   Title: Diagnostic of classical swine fever and its vaccine using recombinant Newcastle disease virus

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VI. Indian Association of Veterinary Microbiologists Immunologists and Specialists in Infectious Diseases (2016)
Title; "Characterization of classical swine fever virus stability at extreme conditions temperature, pH and salt concentration"

VII. Research Conclave 2015
Title: Molecular Characterization of E2 glycoprotein of classical swine fever virus

VIII. Recent Advances in Cancer Biology and Therapeutics -2014
Participant

IX. XXIII National Conferences on Recent Trends in Virology Research in the Omics Era -2014
Title: Molecular characterization of classical swine fever virus following its adaptation in porcine kidney cells

X. Biology and Bioinformatics of Economically important plants and Microbes, University of North Bengal, Darjeeling, West Bengal (2012)
Title: Biopolymers from bacteria to use for bioremediation of toxic heavy metal.

Workshops organized and attended and Volunteer positions held

I. Member of organizing group of Diagnostic Approaches in Virology: An Introductory Workshop, IIT Guwahati (2018)

II. Volunteered at Research Conclave for demonstration of Molecular Microbiology and PCR at IIT Guwahati, in March 2017

III. Participated in Biosafety Awareness Programme and Workshop on "Culture of Responsibility","Pathogen Inventory Management" and "Fundamentals of Working in Biosafety Cabinets" at ICAR – Research Complex for NEH Region, Umiam, Meghalaya in August 2015.

Membership of National and International Bodies

National: Society for Biosafety, India
International: American Society for Microbiology

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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′**AGGCGCGCC***TTAAGAAAAAAT**ACGGG TAGAAGCCACC***ATG**gaaaatataactcaatggaacct-3′ and reverse primer 5′-A**GGCGCGCCTTA**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-

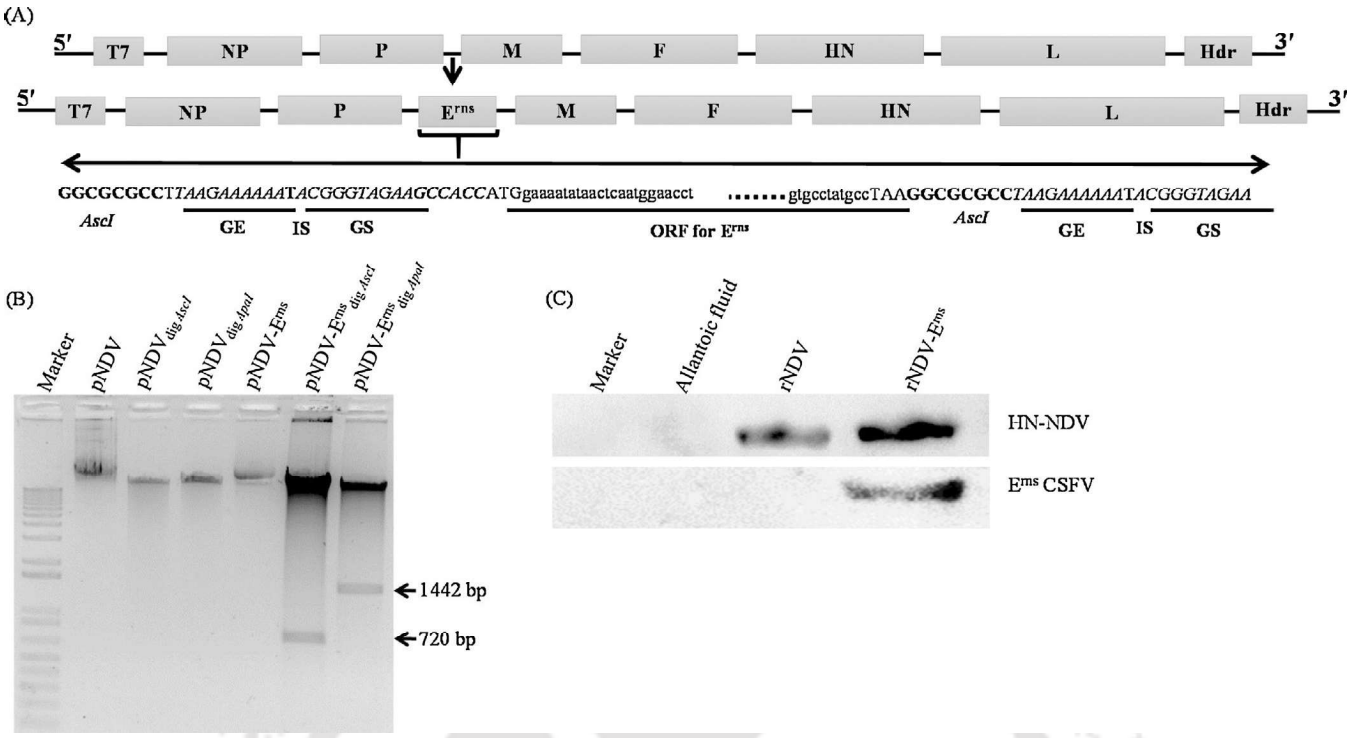

Fig. 1. Construction of recombinant plasmid pNDV-E^{rns}. Schematic representation of upper sense strand DNA of pNDV(T7 promoter, NP- nucleoprotein, P- phosphoprotein, M- matrix protein, F-fusion glycoprotein, HN- hemagglutinin-neuraminidase protein, L-large polymerase protein and Hdr- hepatitis delta ribozyme) and lower sense strand DNA of pNDV-E^{rns}. Transcription cassette encoding E^{rns} was cloned into the *Asc*I site between the P and M genes of the NDV antigenomic cDNA. The E^{rns} ORF has flanking sequence of NDV gene end (GE), an intergenic T nucleotide, and a gene start (GS) (A). pNDV$_{digAscI}$ and pNDV-E$^{rns}_{digAscI}$ represent the digestion of pNDV and pNDV-E^{rns} by*Asc*I to confirm the unique site of *Asc*Iand release of 720 bp (B). In addition, pNDV$_{digApaI}$ and showed release desired fragment confirming the orientation of cloned gene of the E^{rns} in pNDV-E^{rns} (B). Confirmation of NDV and E^{rns} specific protein expression by rNDV-E^{rns} by western blot using monoclonal antibody of HN and polyclonal antibody of CSFV, respectively (C).

HCl (pH 7.0–8.0), or CAPS (pH 9.0–10). The thermo-stability of rE^{rns} was determined by performing the reaction at a temperature range of 15–100 °C. The effect of divalent metal-ion on the RNase activity of rE^{rns} was determined by adding the 10 mM $MgCl_2$ in the reaction mixture with and without of 10 mM EDTA (as a chelator).

2.6. Fluorescence titration of rE^{rns} specific RNAase activity

Fluorimetric titrations of the RNAase activity of rE^{rns} proteins was performed in a quartz cuvette containing 1 μM ethidium bromide (EtBr) solution (Jasco, Japan). The EtBr solution was titrated by the addition of different concentration of tRNA (Sigma–Aldrich, Catalog No. 83850) ranging from 5 μg to 40 μg. The reaction mixture was excited at 510 nm and emission was monitored from 520 to 700 nm using a 2.5/5 nm slit width in 25 mM Tris (pH 6.5) buffer containing 100 mM NaCl.

3. Results

3.1. Construction of pNDV-E^{rns} and recovery of rNDV-E^{rns}

The pNDV-E^{rns} showed the release of 720 bp E^{rns} gene of CSFV upon digestion with *Asc*I (Fig. 1). Further digestion of pNDV-E^{rns} with *Apa*I showed the release of desired 1442 bp fragment (Fig. 1). The nucleotide sequence of the complete pNDV-E^{rns} was found intact without any inadvertent mutation in the NDV genome and E^{rns} gene. The rNDV-E^{rns} was recovered by the established method using the support N, P and L plasmids. The recovered rNDV-E^{rns} was found stable after 20 passages in the 9-day-old chicken embryo and PK-15 cells. The final passaged rNDV-E^{rns} showed amplification of the E^{rns} gene by RT-PCR and nucleotide sequencing. The 20th passaged rNDV-E^{rns} showed a titer of 2^8 (=256) HA unit and 2×10^8 PFU/ml in PK-15. Furthermore, the rNDV-E^{rns} showed positive band of ~44 kDa corresponding to the E^{rns} protein of CSFV on western blot analysis (Fig. 1).

3.2. RNase activity assessment of rNDV-E^{rns}

The isolated cellular RNA was incubated with rNDV-E^{rns} in order to investigate its enzymatic activity. The rNDV-E^{rns} showed complete degradation of the cellular RNA while rNDV as a negative control did not show any effect (Fig. 2). Furthermore, rNDV-E^{rns} showed complete degradation of the cellular RNA 1 h post incubation (Fig. 2B). The RNase activity of rNDV-E^{rns} was slightly higher in the presence of KCl than in NH_4Cl and NaCl (Fig. 2C). Optimum RNase activity of the rNDV-E^{rns} was observed at 150 and 200 mM concentration of KCl. Maximal RNase activity of rNDV-E^{rns} was observed at pH 5–6 (Fig. 2D). The optimum temperature of rNDV-E^{rns} specific RNase activity was calculated at the temperature of 45–50 °C (Fig. 2E). Thermo-stability of rNDV-E^{rns} suggested the loss of its RNase activity beyond 80 °C (Fig. 2F).

3.3. Construction and expression of recombinant E^{rns} expressed in bacteria

The PCR amplified product of E^{rns} after RT-PCR was cloned into pET28a prokaryotic vector flanked with *BamH*I and *Xho*I. The restriction digestion showed the release of 681 bp cloned fragment after digestion with *BamH*I and *Xho*I (Fig. 3). Furthermore, the rE^{rns} protein gene was found intact after its nucleotide sequencing. The rE^{rns} expressed in BL21 cells as a fusion protein with His tag produced a ~35 kDa protein (Fig. 3). The average yield of the different fraction of the rE^{rns} protein was 0.8 mg/ml. Finally, purified fraction of the rE^{rns} protein showed a band of ~35 kDa on western blot (Fig. 3).

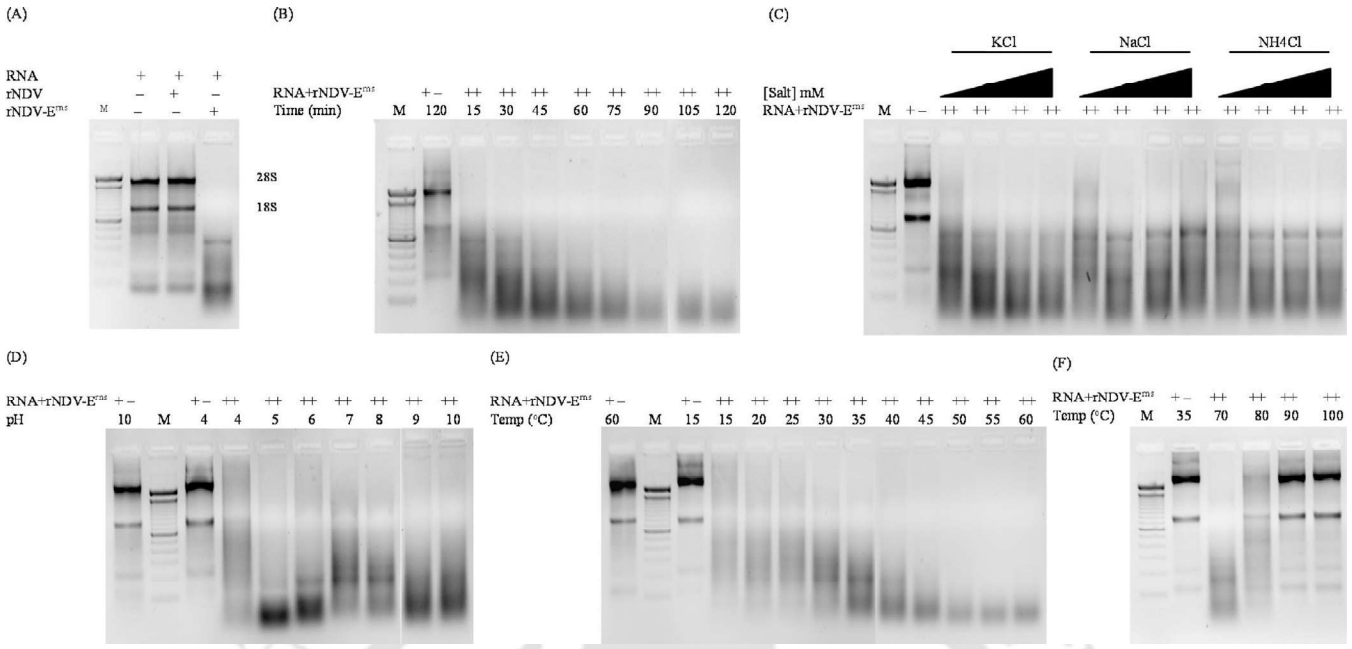

Fig. 2. Assessment of rNDV-E^{rns} enzymatic activity. RNase activity of rNDV-E^{rns} was specific to E^{rns} (A). Agarose gel showing RNase activity of rNDV-E^{rns} at the different time interval (B). Furthermore, agarose gel showing RNase activity of rNDV-E^{rns} in presence of various salts under concentration of 50–200 mM (C) and different pH conditions (D). Agarose gel showing RNase activity of rNDV-E^{rns} at the different temperature from 15 to 60 °C (E). Effect of preheats treatment of rNDV-E^{rns} showing impaired enzymatic activity above 80 °C (F).

3.4. RNase activity assessment of prokaryotic expressed E^{rns}

The isolated cellular RNA was incubated with rE^{rns} protein to observe its enzymatic activity. The rE2 protein of CSFV and RNaseA (NEB, USA) was used as a negative and positive control, respectively. The rE^{rns} and RNaseA showed degradation of the cellular RNA while rE2 did not show any effect (Fig. 4A). Furthermore, the rE^{rns} protein showed its maximum RNase activity at 2 h post incubation (Fig. 4B). The RNase activity of rE^{rns} was found higher in the presence of KCl than in NH_4Cl and NaCl (Fig. 4C). In addition, NaCl at a concentration of 150 and 200 mM inhibits the RNase activity of the rE^{rns} protein. The rE^{rns} protein exhibited RNase activity from pH 4–10 (Fig. 4D). However, maximum activity was observed at pH 4–5 and decreased gradually beyond pH 5. Maximum RNase activity of the rE^{rns} protein was recorded at 45–50 °C (Fig. 4E). However, the rE^{rns} protein did not show any effect during incubation with RNA above 90 °C (Fig. 4F). The RNase activity of the rE^{rns} protein was inhibited by divalent metal ions. Zn^{2+} ions was found to be the most potent inhibitor of its RNase activity (Fig. 4G). Moreover, chelating metal ions (Mg^{2+}), restores the RNase activity of the rE^{rns}. On the other side, Mg^{2+} ion was found to be the weakest inhibitors among all (Fig. 4H).

3.5. Fluorimetric titrations of the E^{rns}

The change of fluorescence intensity at a given EtBr concentration with increasing concentration of RNA gave a hyperbolic graph (Fig. 5). The maximum fluorescence intensity of the EtBr after binding with RNA was observed at 581 nm. The degradation of RNA was monitored with the change in the fluorescence intensity of emission. The rE^{rns} in 1400 nM concentration showed 50% reduction in RNA.

4. Discussion

CSFV is an important pathogen of swine and its biology has been explored in order to make its proper diagnostics and vaccine [42–44]. Although RNase activity of the E^{rns} protein of CSFV has been discussed using yeast expression system to a certain extent, its activity using a mammalian host system has not been explored [45]. Our study of rNDV expressing E^{rns} protein of CSFV would be an ideal way to express its native form. Moreover, the E^{rns} expressed as part of the rNDV structural protein allowed to retain its folding by virtue of its stoichiometry. It has been shown earlier that protein expressed as a part of NDV will have high functionality [46,47].

Presumably, rNDV-E^{rns} will have native conformation and

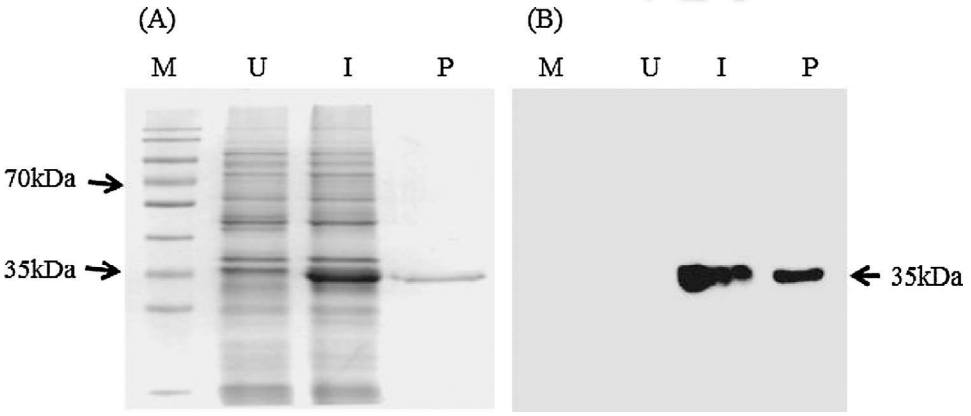

Fig. 3. Expression analysis of recombinant E^{rns} protein in bacteria. The SDS-PAGE analysis of the E^{rns} protein of CSFV expressed in pET28a prokaryotic expression vector (A). M: protein molecular weight marker; U: uninduced recombinant pET28a containing E^{rns} protein gene; I: expression of protein in recombinant pET28a containing E^{rns} protein gene after 3 h of 0.5 mM IPTG induction; P: purified recombinant Erns protein. Western blot analysis showing the presence of recombinant E^{rns} protein using anti-His antibody (B).

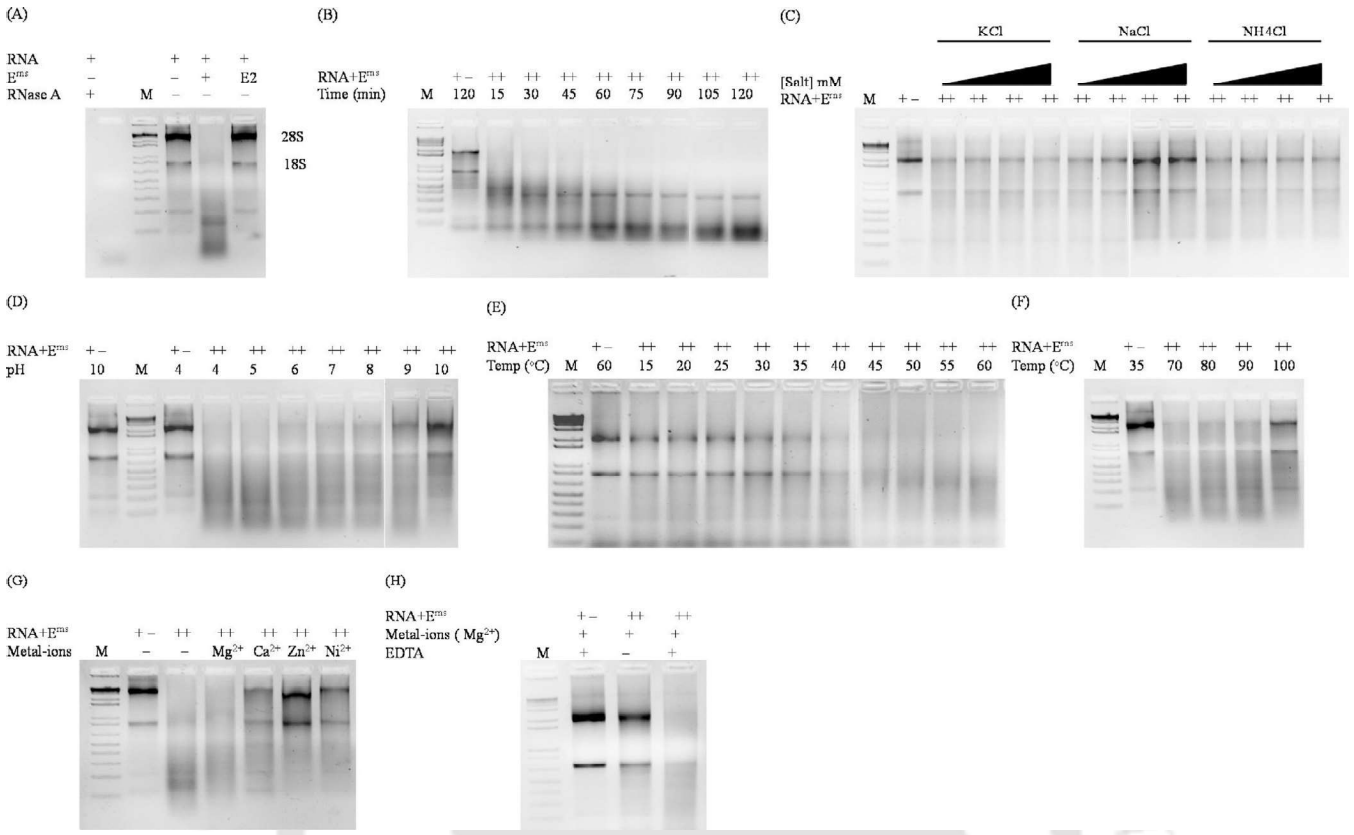

Fig. 4. Enzymatic activity assessment of recombinant E^{rns} protein. RNase activity is specific to recombinant E^{rns} protein and not by any other bacterial protein (A). Agarose gel showing RNase activity at the different time interval (B), in presence of various salts under concentration of 50–200 mM (C), and at different pH (D). Agarose gel showing RNase activity of bacterial expressed E^{rns} at different temperature (E). Effect of pre-heating treatment of recombinant E^{rns} showing its impairment above 90 °C (F). Effect of divalent metal ions (G) and its chelation (H) on RNase activity of recombinant E^{rns}.

glycosylation (Supplementary Fig. 1). It is well established that inappropriate glycosylation can impact the enzymatic activity of a protein [48–50]. The rNDV-E^{rns} showed a RNase activity with the cellular RNA suggesting the functional activity of the expressed recombinant protein. The rNDV-E^{rns} showed its optimum RNase activity at a temperature range of 45–50 °C and pH range of 4–7 consensus with the earlier report [19,21].

To further strengthen the findings, we expressed the rE^{rns} in a prokaryotic expression system. In contrary to the earlier report, our study of prokaryotic expressed rE^{rns} showed RNase activity with the cellular RNA [51]. The pattern of the protein folding largely depends on the condition of its expression and the accessory tag used [52]. The difference in our results might be due to the expression vector used for the protein production and its folding, which may have exposed its functional domain. Furthermore, the rE^{rns} showed positive *fluorimetric* results suggesting the functional protein. It has been shown that RNA binding with EtBr could be used to assess the RNase activity [53]. Our results showed that fluorescence intensity of RNA bound EtBr decreased with increasing concentration of rE^{rns} suggested RNA degradation in presence of rE^{rns}. Moreover, the enzymatic activity of the E^{rns} expressed as a viral recombinant protein was quite higher than the one expressed by bacteria. We believe that expression of rNDV-rE^{rns} will have enhanced activity because of its native and glycosylated forms, which was absent in its bacterial form. However, the thermal stability of the rNDV-rE^{rns} was lower as compared to a bacterial expressed form of rE^{rns}. Our results suggest that the stability of the rE^{rns} activity could be modulated by its glycosylation. It has been proposed that metal ions could modulate the RNase activity of the recombinant protein [54]. In our study, the metal ion showed minimal effect on the RNase activity of the rE^{rns} corroborating to the earlier findings. However, a higher concentration of the Mg ions (> 10 mM) showed inhibitory effect on the RNase activity of the recombinant E^{rns}.

It has been shown that the RNase activity of E^{rns} leads to immune suppression of the host by inducing apoptosis of lymphocytes [22]. The E^{rns} protein of CSFV has been explored for its diagnostic potential [55,56]. It will be interesting to explore the immunobiology of CSFV using rNDV-E^{rns}. The rNDV-E^{rns} will express the recombinant protein through normal infection in pigs. Moreover, infection of rNDV-E^{rns} will give us a better insight regarding its biology as compared to other expressed forms.

The proposed methodology will be an ideal way of producing a highly soluble recombinant protein with its native conformation and function. Moreover, a single embryonated chicken egg will be able to produce high amount (~3 mg) of recombinant protein without involving any instrument. In addition, the methodology will be an ideal, rapid and highly cost-effective way of producing recombinant enzymatic protein. The lentogenic strains of NDV have been routinely used for vaccine production and will be an ideal vector to produce recombinant proteins or enzymes.

Conflict of interest

The authors declare no conflict of interest.

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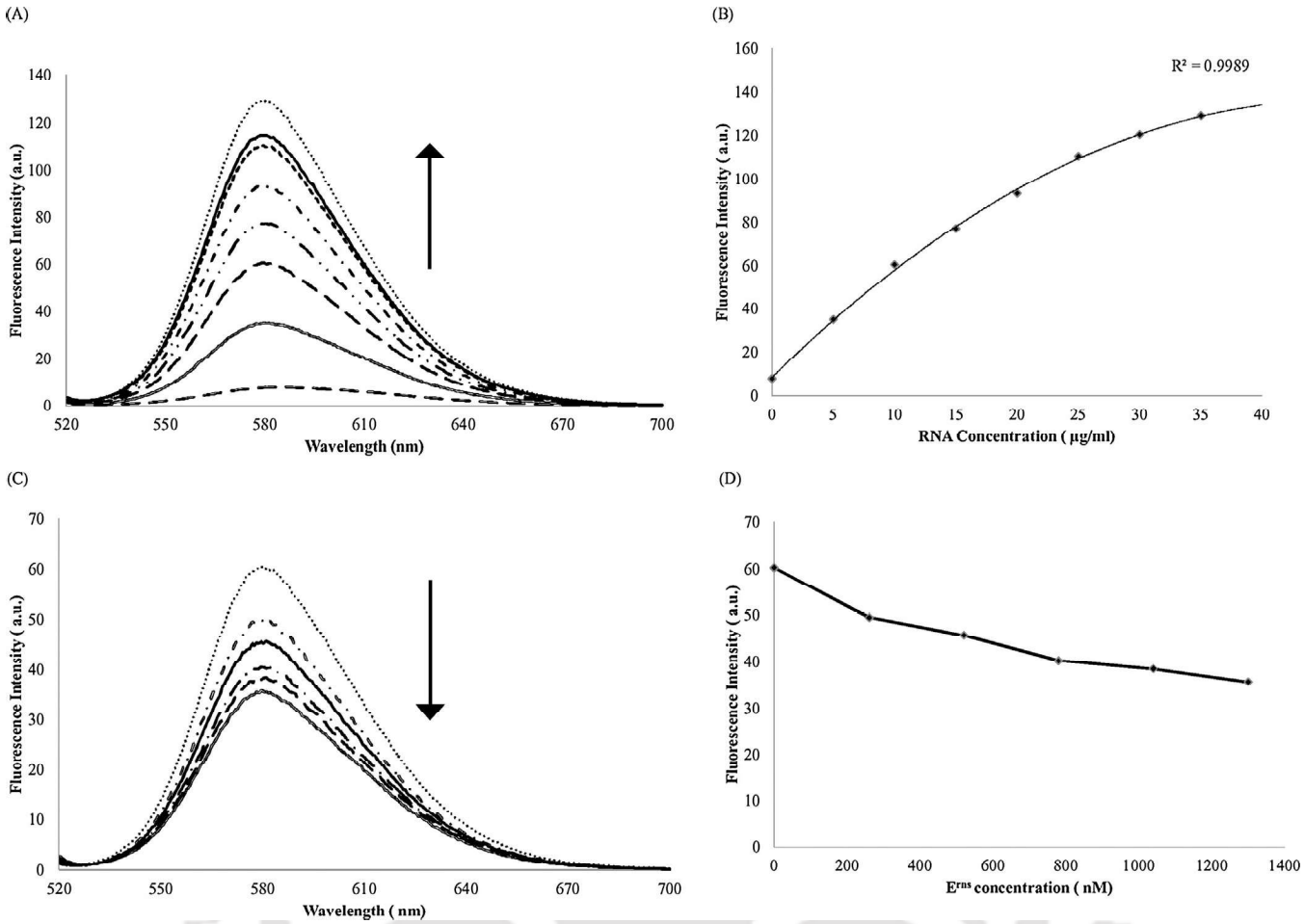

Fig. 5. RNase activity of recombinant E^{rns} protein by spectrofluorimeter. Fluorescence spectra of EtBr (1 µM) in the presence of RNA (0–35 µg/ml); the arrow indicates increasing concentrations of RNA (A). Fluorescence intensity of EtBr (1 µM) in the presence of varying concentrations of RNA on excitation at 510 nm and emission at 581 nm (B). Fluorescence spectra of EtBr–RNA (1 µM) in the presence of recombinant E^{rns} protein (0–1300 nM); the arrow indicates increasing concentrations of RNA (C). The relationship between the changes in fluorescence intensity of the EtBr–RNA system with increasing concentrations of recombinant E^{rns} protein (0 to1300 nM) (D).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.procbio.2017.12.001.

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Short communication

Development of single dilution immunoassay to detect E2 protein specific classical swine fever virus antibody

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ABSTRACT

Classical swine fever virus (CSFV) is the causative agent of a highly contagious disease in swine. The disease is endemic in different parts of the world and vaccination is the only way to protect pigs from CSFV infection. The virus surface protein E2 is the major immunogenic protein eliciting protective immunity against CSFV infection in swine. The whole virus antigen cannot differentiate CSFV from other pestiviruses as it cross reacts with border disease and bovine viral diarrhoea viruses. Commercial available ELISA is based on the whole CSFV particle and can lead to false positive results. Moreover, the available commercial ELISA is not cost effective. In the present study, a recombinant E2 protein based single serum dilution ELISA was developed which showed enhanced sensitivity, specificity and accuracy as compared to commercial CSFV detection ELISA. The recombinant E2 protein based ELISA could be an alternate to existing diagnostics against CSFV infection in pigs.

Classical swine fever virus (CSFV) is the causative agent of a highly infectious disease in swine. The classical swine fever (CSF) or hog cholera can cause huge economic impact to the pig industry worldwide (Moennig, 2000). It affects both domestic and wild pigs with varying degrees of pathogenicity. Although CSF is distributed throughout the world, it has mainly been reported from Asia, parts of Africa, Central and South America and Europe (Artois et al., 2002; Barman et al., 2014; Flores-Gutierrez and Infante, 2008). Frequent outbreaks of CSF have been reported from different parts of the world, including Belgium (1990–94), Germany (1993–2000), Italy (1995–97) and the Netherlands (1997). Recently, CSF outbreaks have been reported from Madagascar, Singapore, Laos, Lithuania, Myanmar, Colombia, and Republic of Korea (Ji et al., 2015). However, the disease has been eradicated from Australia, North America, and New Zealand (Anonymous, 2008).

CSFV belongs to the family *Flaviviridae* under genus *Pestivirus*. CSFV is an enveloped virus having a positive strand RNA with an approximately size of 12.5 kb and comprises of a single large open reading frame (ORF) (Meyers and Thiel, 1996). The genome of CSFV is flanked by two untranslated regions (UTRs) flanking the entire ORF which encodes a polypeptide of approximately 3900 amino acids (Meyers et al., 1996). This polyprotein gives four structural (C, Erns, E1, E2) and eight nonstructural proteins after processing by the cellular and viral proteases (Npro, P70, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Lowings et al., 1996; Meyers and Thiel, 1996). Npro protein is a non-structural protein, which functions as a cysteine proteinase (Bauhofer et al., 2005). Erns is a glycoprotein secreted from the cells infected by CSFV and is known to inhibit the interferon induction by the host cell (Matzener et al., 2009; Weiland et al., 1999). E1 is a type I trans-membrane protein involved in the adsorption of virus to the host cells (Fernandez-Sainz et al., 2009). CSFV encodes another small hydrophobic protein P7, which is flanked by sequences that are recognized by signal peptidase and is essential for the production of infectious virus (Moser et al., 1999).

E2 is an envelope glycoprotein present on the surface of CSFV and is important to induce host immune response during infection (Qi et al., 2009; Zhang et al., 2006). The E2 protein contains conserved antigenic determinant regions and it is the major immunogenic protein eliciting protective immunity against CSFV infection in swine (Greiser-Wilke et al., 1990; Rumenapf et al., 1991; van Rijn et al., 1996). E2 protein contains two linear B cell epitopes YYEP and TAVSPTTLR spanning towards its carboxy and amino terminus, respectively. The amino acid sequence motif YYEP is specific to pestivirus while TAVSPTTLR is specific to CSFV (Lin et al., 2000; Yu et al., 1996). In addition, E2 protein is accompanied with four relatively independent antigenic domains (A, B, C and D). The domain A has three subdomains (A1, A2 and A3). The E2

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is frequently used to design DNA vaccines against CSFV by different research groups (Beer et al., 2007; Bouma et al., 1999; Qi et al., 2008). Structurally, the E2 protein forms a homodimer during entry and heterodimer with E1 while attachment of the viral particles to the cell (Zhang et al., 2006).

In epidemiological surveys, detection of virus specific antibodies in serum samples is important in order to monitor the circulation of wild CSFV in population. Neutralizing assay is the test of choice to detect CSFV infection in the laboratory. However, detection of neutralizing antibody is time consuming, needs skill and well set up cell culture laboratory. Development of single dilution indirect ELISA can be a convenient alternative tool to detect CSFV specific antibody in pig sera (Li et al., 2013; Yang et al., 2012) using complete E2 protein as detecting antigen. Single serum dilution could be better than serial dilution method because it requires less time, chemical and plasticware making it a cost effective assay. Here we are reporting the expression of complete E2 protein using the bacterial expression system. The bacterial expressed E2 protein showed efficient binding with monoclonal antibody and with the serum collected from the field outbreaks. The study will be useful in designing an efficient diagnostics against CSFV infection.

The porcine kidney cells (PK-15) were procured from ATCC (Manassas, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and essential antibiotics at 37 °C in 5% CO_2. The CSFV vaccine strains were procured from college of veterinary science, Guwahati, India. The virus was grown in PK-15 cells using standard protocols (Hulst et al., 2000). The infectious virus particles were recovered by repeated freeze and thaw followed by filtering the extracted supernatant through 0.22 μm membrane filter. The stock of the virus was stored at −80 °C for subsequent use. Titration of the virus was done in 96-wells microtitre plate containing PK-15 cells. Vaccine virus was diluted ten-fold in DMEM containing 10% calf serum into each of the five wells in a 6-well plate. The titer of virus stock was calculated by immunoperoxidase assay using CSFV monoclonal antibody after 72 h post-infection as described earlier (Mittelholzer et al., 1998).

Viral RNA was extracted using TRIzol (Invitrogen, USA) according to the standard protocol. The cDNA was synthesized from extracted RNA using superscript II RT (Invitrogen, USA) and gene specific reverse primer (5′ CCG*CTCGAG*TCAACCAGCGGCGAGTTGTTCTG 3′) designed from available GenBank sequence (Accession number NC_002657). The E2 gene sequence was PCR amplified using E2 forward (5′ CG*GAATTC*ATGCGGCTAGCCTGCAAGGAAGATTAC 3′) and E2 reverse (5′ CCG*CTCGAG*TCAACCAGCGGCGAGTTGTTCTG 3′) primers. Underline sequences are complementary to the CSFV genome and italics sequences are the restriction site *Eco*RI and *Xho*I, respectively. The amplified PCR products were purified by QIAquick gel extraction kit (QIAGEN, Germany) and sequenced by BigDye terminator v 3.1 matrix standard kit and 3130xl Genetic Analyzer Data Collection software v3.0 (Applied Biosystems, USA). The data extracted from analyzer was analyzed by DNAstar software (DNASTAR Inc, USA).

The complete E2 protein gene was cloned into pET28a prokaryotic expression vector (Novagen, Germany) flanking *Eco*RI and *Xho*I restriction sites. The pET28a has His tagged at the N terminal which was used to purify the E2 protein by affinity chromatography. The integrity of the E2 gene after cloning was confirmed by sequencing. The pET28a containing complete E2 gene was transformed into *Escherichia coli* BL21 (DE3) pLysS cells (Novagen, Germany) and induced by 1 mM isopropyl-β-*D*-thiogalactoside (MBI Fermentas, Germany). The fusion protein was extracted after induction of transformed BL21 cells for 4 h at 37 °C and purified by affinity chromatography containing Ni-NTA (Invitrogen, USA). 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 dialyzed using 10% glycerol in PBS. The concentration of the purified protein was determined by modifying Lowry's Protein assay kit according to the manufacturer's protocol (Pierce, USA). The expression of the recombinant E2 protein was further confirmed by SDS-PAGE and western blot using an anti E2 monoclonal antibody WH 303 (Wensvoort et al., 1986).

Two rabbits were immunized subcutaneously with 0.5 mg of purified recombinant E2 protein emulsified in Freund's complete adjuvant (Sigma, USA) for the preparation of polyclonal antibodies. Subsequent booster was given by purified recombinant E2 protein with Freund's incomplete adjuvant (Sigma, USA) for 2 occasions at 14 days interval. The sera sample was collected 14 days after final injection and stored at −80 °C. The antibody against recombinant E2 protein was confirmed by western blot using goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, USA) as well as by commercial CSFV kit (HerdChek, IDEXX, USA). A total of 210 pig serum samples were screened for the CSF infection. Blood samples of suspected pigs were collected under aseptic condition and serum were separated after centrifugation at 1500 × *g* for 20 min, and stored at −20 °C until use. Additionally serum samples were screened negative for swine flu and porcine reproductive and respiratory syndrome using a commercial kit (PRRS X3 and influenza A kit from IDEXX, USA).

96-well flat bottom polystyrene plates (Greiner, USA) were coated with recombinant E2 protein (3 μg/well) using nitrate buffer (pH = 9.6) and incubated at 4 °C overnight. Plates were washed with phosphate buffer saline containing Tween-20 (PBST) and blocked with 5% lactalbumin hydrolysate for 1 h at 37 °C. Optimal concentration of E2 protein was determined by the checkerboard titration method (Robinson et al., 1985). Pig serum samples were initially diluted 10 times and then diluted serially and incubated at 37 °C for 1 h. The plates were then washed with PBST, and incubated with 100 μl of the HRP-conjugated anti-pig antibody raised in rabbit (Pierce, USA) for another 1 h at 37 °C temperature. The plates were washed and the E2 protein binding with serum samples was detected with 100 μl of TMB (Invitrogen, USA) for 15 min at 37 °C temperature. The enzymatic reaction was stopped by 100 μl of 2 M H_2SO_4, and plates were read at 450 nm in a microtitre plate reader (Biotek, USA). Any serum sample showing an OD above the mean +3 standard deviation of the negative wells was considered positive. The negative serum samples were used to construct positive-negative threshold (PNT) baseline as shown earlier (Snyder et al., 1983). Similarly the positive curve was also plotted. The absorbance of the test sample dilutions was calculated using the formula:

$$\text{SPratio} = (\text{ODofsample} - \text{ODofnegative})/(\text{ODofpositive} - \text{ODofnegative})$$

The PNT line was calculated using negative serum samples which were screened negative by commercial kit. The collected negative serum samples were diluted and resultant OD values were plotted against dilution. The resultant PNT line was used to find out the titer for the known positive serum samples by the subtraction method as described earlier (Snyder et al., 1983). The OD values obtained for every logarithmic dilution was compared with observed titer and the highest correlation coefficient was selected to calculate the titer from that dilution. The constants like slope and intercept were calculated by the scatter plot as described earlier (Snyder et al., 1983).

The sensitivity, specificity and accuracy of the single dilution sera in comparison to the commercial CSFV diagnostic are determined using following formulae.

$$\text{Sensitivity} = (x/x + y) \times 100$$

where, 'x' is the number of sera positive by commercial CSFV diagnostic ELISA and single dilution ELISA; 'y' is the number of sera

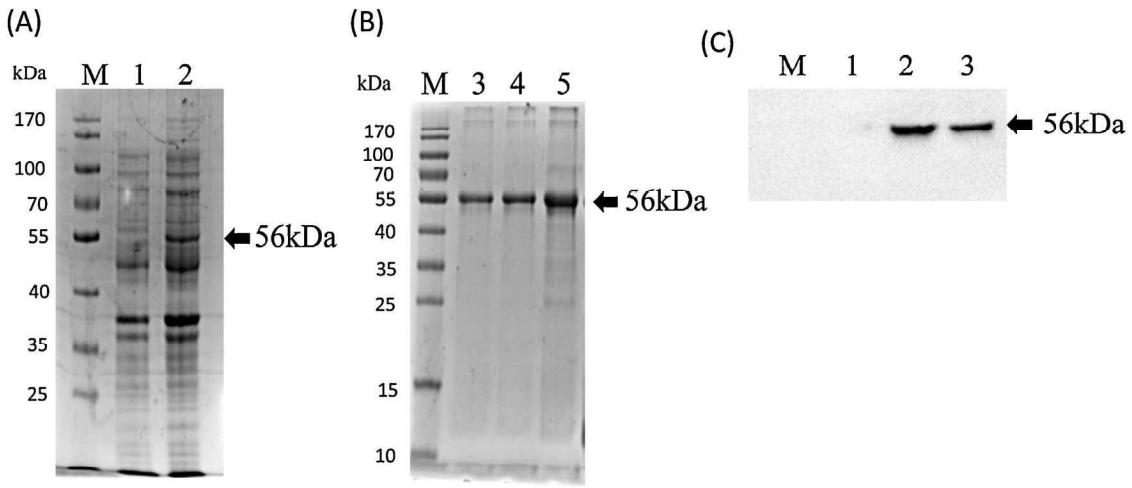

Fig 1. Expression analysis of recombinant E2 protein of classical swine fever virus (CSFV). The SDS-PAGE analysis of the E2 protein of CSFV expressed in pET28a prokaryotic expression vector. M: protein molecular weight marker; Lane 1: uninduced recombinant pET28a containing E2 protein gene; lane 2: expression of protein in recombinant pET28a containing E2 protein gene after 2 h of 0.5 mM IPTG induction (A). Lane 3, 4, and 5: different fractions of purified recombinant E2 protein (B). Western blot analysis of recombinant E2 protein using polyclonal antibody raised in rabbits. Lane1: pET28a vector control; lane2: purified recombinant E2 protein lane3: CSFV purified virus control (C).

negative by our test ELISA and positive by commercial CSFV diagnostic ELISA.

$$\text{Specificity} = (\alpha/\alpha+\beta)\times 100$$

where, 'α' is the number of sera negative by commercial diagnostic ELISA and single dilution ELISA; 'β' is the number of sera positive by single dilution ELISA and negative by commercial diagnostic ELISA.

$$\text{Accuracy} = (x+\alpha/x+y+\alpha+\beta)\times 100$$

The PCR amplified product of CSFV E2 protein was successfully cloned into pET28a vector and showed release of desired fragment upon digestion with *Eco*RI and *Xho*I. Furthermore, the sequencing of the E2 protein showed intactness of the nucleotide sequence. The recombinant E2 protein expressed in BL21 cells as a fusion protein with His tag was about 56 kDa in size (Fig. 1A and B). The average yield of the recombinant E2 protein was 70 mg/l of the bacterial culture used for its purification. The recombinant E2 protein showed the corresponding band of 56 kDa in western blot using raised polyclonal rabbit sera (Fig. 1C). The ELISA results and indirect immunoperoxidase assay confirmed the specificity for raised polyclonal antibodies (data not shown).

The calculated OD values were plotted on Y-axis against different serum dilutions in X-axis. The point where the sample line cuts the PNT line is taken as titer of the sample. The PNT baseline with different positive serum samples is presented diagrammatically and single serum dilution was calculated by linear regression (Fig. 2). The checker board titration of purified recombinant E2 protein showed 50 ng/well of protein being optimized for working range. The PNT curve for the calculation of the sample serum titer was obtained manually. ELISA using the single serum dilution method was performed using 50 serum samples. The correlation coefficient of 0.9349 was calculated at 1:80 dilutions that were more than the calculated value for all other dilutions. Thus, 1:80 dilution of serum samples was further considered to predict the titre of remaining serum samples. The slope and intercept were 3.8246 and 0.0958, respectively. The observed and predicted OD for all the serum samples were calculated using the regression equation, Observed OD = 3.8246(predicted OD) +0.0958. A linear relation was observed between the predicted and observed titres (Fig. 3).

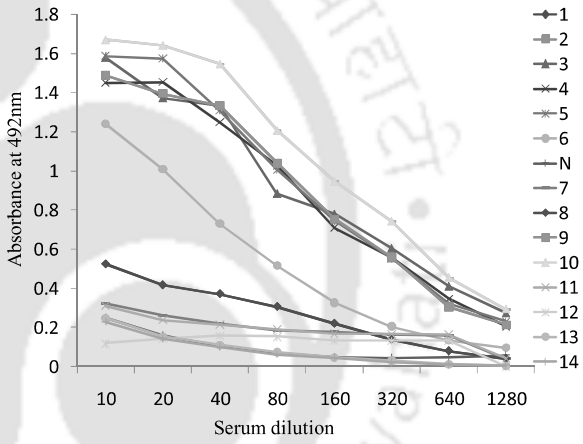

Fig. 2. The standard serial dilution method of determining observed CSFV ELISA antiserum titres from corrected absorbance with positive-negative threshold (PNT) baseline. N represents the PNT baseline while other lines show the different positive and negative serum samples.

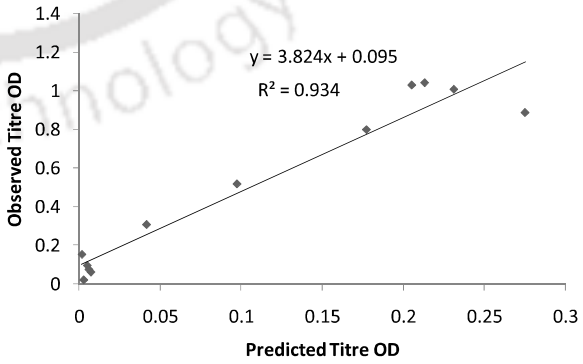

Fig. 3. The relationship between the observed antibody titres of the serum samples obtained after serial dilution and their corresponding predicted antibody titres obtained from a single dilution ELISA at a 1:80 dilution.

The sensitivity, specificity and accuracy of the assay relative to the commercial diagnostic ELISA are shown in Table 1. The sensitiv-

Table 1
Relative sensitivity, specificity and accuracy of the developed recombinant E2 protein based ELISA to detect CSFV antibodies commercial CSFV ELISA assay as a reference standard.

	Commercial CSFV ELISA		
Single serum dilution ELISA	+ve	−ve	Total
+ve	166	3	169
−ve	4	37	41
Total	170	40	210

Sensitivity: (166/170) × 100 = 97.65%.
Specificity: (37/40) × 100 = 92.50%.
Accuracy: (200/210) × 100 = 95.24%.

ity, specificity and accuracy of recombinant E2 protein based ELISA was calculated 97.65%, 92.50%, and 95.24%, respectively.

CSFV shares antigenic epitopes with other pestiviruses such as border disease virus (BDV) and bovine viral diarrhea virus (BVDV) (Beer et al., 2007; Moennig, 2000). Although both BDV and BVDV are species specific, they may infect pigs making the diagnosis of CSFV difficult in field condition (Lin et al., 2000). Eradication of CSFV from swine requires serological methods which are rapid and simple to perform. Moreover, the test should clearly differentiate between CSFV and other pestiviruses. In addition, the test should also detect antibodies early during the CSFV infection. In view of the above facts expression of CSFV specific E2 protein is necessary to differentiate CSFV from BDV and BVDV. In the present study, the E2 protein of CSFV was expressed from a bacterial expression system and the purified protein was used as an antigen for diagnostic ELISA. Recombinant protein-based serological tests are considered to have higher sensitivity and specificity as the target antigen is immunodominant and devoid of any non-specific moieties present in whole cell preparations. We have successfully cloned and expressed the E2 protein of CSFV using the bacterial expression system. Although the actual biological activity of E2 being a glycoprotein in the bacterial expressed form may not be justifiable in the absence of post translational modification, its use in the diagnostics may have an added advantage. Our result showed high yield of E2 protein (70 mg/l) from bacterial culture suggests its efficient production. Our study showed that E2 protein based ELISA can be used for rapid and efficient screening of a large number of serum samples, especially during assessment of vaccination status of pigs involving large herd.

The sensitivity and specificity of the E2 protein based ELISA was 97.65% and 92.50%, respectively, relative to the commercial ELISA. The close correlation obtained between single serum and commercial ELISA titre showed similar trends in exposure to CSFV positive serum. However, the ELISA appears to be more sensitive than the commercial ELISA test and is able to detect antibody activity against CSFV in field samples that is not detectable by the commercial ELISA (Table 1). The ELISA reported in the paper using E2 protein would help to overcome some of the economic, technical and statistical constraints of using this assay as a rapid serological assay against CSFV infection in pigs. The recombinant E2 protein-based single serum dilution ELISA for the detection of antibodies developed in this study was shown to be sensitive, specific and accurate as compared to the available commercial ELISA. The study will help us to understand the use of viral surface protein in diagnostics of CSFV. These results demonstrate the potential benefit of a simple, specific ELISA for anti-E2 antibodies that may have diagnostic value for the pig industries. It will be interesting to explore the possibility of using other immunogenic proteins of CSFV for its diagnosis.

Conflict of interest

The authors declare that they have no conflict of interest.

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REPORT

Molecular characterization of E2 glycoprotein of classical swine fever virus: adaptation and propagation in porcine kidney cells

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Abstract Classical swine fever virus (CSFV) is the causative agent of a highly contagious disease, hog cholera in pigs. The disease is endemic in many parts of the world, and vaccination is the only way to protect the animals from CSFV infection. The lapinized vaccine strains are occasionally not protective because of animal to animal passage, inadequate vaccination strategy, suboptimal vaccine dose, and emergence of new variants. The surface glycoprotein E2 of CSFV is a major antigenic determinant and can modulate the disease outcome in pigs. In the present study, we characterized the CSFV in porcine kidney cells. The CSFV vaccine strains showed enhanced replication following 15 passages in porcine kidney cells. Nucleotide sequence analysis of the E2 protein gene of the cell culture-adapted vaccine strain of CSFV showed a mutation in putative amino acid sequences that are identical to its virulent counterpart. The study suggests the possibility of exaltation in vaccine strains following its adaptation in host cells and paves the way for a further exploration of the biology of its outbreak.

Keywords Classical swine fever virus · E2 glycoprotein · Lapinized vaccine · Pig kidney cells

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Report

Classical swine fever (CSF) is a highly contagious viral disease that mainly affects domestic pigs, wild boar, and pygmy hog (Dahle and Liess 1992; Artois *et al.* 2002; Barman *et al.* 2012). It causes huge economic losses to the pig industry worldwide. Although CSF has been eradicated from some parts of the world, a reservoir is maintained in wild boar populations and the virus can be reintroduced into domestic pigs, resulting in fresh outbreaks and the spread of the disease. The disease is endemic in India, and regular outbreaks have been reported in the northeastern part of the country which has adjoining borders with Bhutan, China, Myanmar, and Bangladesh (Barman *et al.* 2010). Animal migration from adjoining countries is very common and contributes to outbreaks in vaccinated herds. The mean prevalence of classical swine fever virus (CSFV) antibodies was 63.3% (376/594), whereas the viral antigen prevalence was 76.7% (220/287) from 594 samples collected from 12 states (Rahman 2011). Although the lapinized C strain vaccine is widely used, the high prevalence of CSFV antibodies suggests that the disease is endemic in India and is a major threat to the pig industry. However, CSFV-specific antibodies in a flock could be due to vaccination. Moreover, vaccination outbreaks were also recorded on many occasions, raising a question on the efficacy of the

vaccine (Suradhat and Damrongwatanapokin 2003; Ji *et al.* 2014). Moreover, the efficacy of the vaccine depends on vaccination strategy, suboptimal vaccine dose, and hygienic measures (Elbers *et al.* 2001; Klinkenberg *et al.* 2003). In addition, animal to animal passage can modulate the disease outcome because of the mutation in the E2 gene that can alter the host immune response (Wehrle *et al.* 2007; Tamura *et al.* 2012; Fahnoe *et al.* 2014).

CSFV belongs to the genus *Pestivirus* under the family *Flaviviridae* (Thiel *et al.* 1993). The genome of CSFV is a positive-strand RNA of approximately 12.5 kb in length and is comprised of a single large open reading frame (Meyers *et al.* 1989; Becher *et al.* 1994). It is believed that the viral genes are translated as a large polyprotein that undergoes subsequent proteolytic processing by cellular and viral proteases (Rumenapf *et al.* 1991). Four structural genes, namely core protein (C), E^{rns}, E1, and E2, are found in the infectious virion. The C protein has RNA chaperone activity, and it binds to viral RNA in order to facilitate its packaging into the virion (Ivanyi-Nagy *et al.* 2008; Murray *et al.* 2008). The envelope of the CSFV contains three glycoproteins E^{rns}, E1, and E2 (Thiel *et al.* 1991). The E2 protein in CSFV-infected animals is a major immunogen and contains conserved antigenic determinant regions (van Rijn *et al.* 1996; van Rijn 2007). The E2 protein plays an important role in viral adsorption to host cells along with E^{rns} and E1 (Hulst and Moormann 1997; Wang *et al.* 2004). Virus neutralizing antibodies are raised against E2 in CSFV-infected animals (de Smit *et al.* 2000; Qi *et al.* 2009). A cell line from a natural host is generally permissive for pestivirus replication in vitro, but considerable differences have been observed in its replication efficiency (Roehe and Edwards 1994; Flores *et al.* 1996). Moreover, mutations in the E2 gene of vaccine strains are responsible for the adaptation of pestiviruses into a permissive cell line (Liang *et al.* 2003). The putative cell surface receptor for pestiviruses was identified as CD46, and its interaction with the envelope protein is important for endocytosis of the virion (Schelp *et al.* 2000; Maurer *et al.* 2004; Krey *et al.* 2005; Tews and Meyers 2007). The E2 gene in CSFV encodes for a protein 373 amino acids long of 51 to 55 kDa size based on the level of glycosylation. The E2 protein has four antigenic domains, namely A, B, C, and D, within the N terminus (Wensvoort 1989). The E2 protein forms a homodimer and heterodimer with the E1 protein during virus entry and attachment to the host cells (Zhang *et al.* 2006). The sequential epitopes of the E2 protein elicit neutralizing antibodies, which direct protective immunity. Therefore, E2 is explored as a candidate for DNA vaccines in order to prevent CSFV infection (Bouma *et al.* 1999; Qi *et al.* 2008; Qi *et al.* 2009). Variation in E2 proteins can lead to the emergence of a new variant of CSFV (Chen *et al.* 2010; Leifer *et al.* 2012). No information is available regarding changes occurring in the lapinized CSFV strain following its adaptation in a cell culture system.

In the present study, we have analyzed the mutations in the E2 protein gene of live attenuated CSFV vaccines used in the field condition at the level of nucleotides as well as amino acid residues following its adaptation in porcine kidney cells. The study will help us in understanding the biology of the CSFV surface protein following its adaptation in porcine cells. Moreover, knowledge regarding mutations in the surface glycoprotein and its effect towards increased binding efficiency with the cell surface receptor can be a useful tool to design a better vaccine using a reverse genetics system.

The porcine kidney cells (PK-15) were procured from the ATCC (Manassas, VA). The cells were maintained in Eagle's minimal essential medium (EMEM) containing 10% fetal calf serum (Invitrogen, Grand Island, NY) tested free for BVDV at 37°C in 5% CO_2. Three lapinized vaccine viruses, namely IVRI (Indian classical strain), Assam lapinized (Assam), and virus isolated from vaccinated pigs (local strain), were procured from the Department of Microbiology, College of Veterinary Science, Khanapara, Guwahati, India. The sequences of the three lapinized vaccine strains regularly used under field conditions were extracted from GenBank (accession numbers EU857642 (Indian classical strain), EU567078 (Assam strain), and KP195022 (local strain)).

The CSFV strains were passaged in PK-15 cells in 80% to 90% confluency blindly for 15 passages. For CSFV infection in PK-15 cells, 1 ml of virus inoculum treated with antibiotic and filtered through a 0.25-μ syringe filter was allowed to adsorb on the monolayer for 60 min. The EMEM containing 5% fetal calf serum tested free of pestivirus was overlaid after draining the virus inoculums from PK-15 cells. The cells are further incubated at 37°C under 5% CO_2 for 5 d. The infected PK-15 cells were freeze-thawed for three cycles and clarified at 3000 rpm, and the supernatant was collected as virus inoculum for the second passage and stored at −80°C.

The replication of CSFV strains in the PK-15 cells was analyzed by immunofluorescence (IF) and immunoperoxidase (IP) staining using the WH303 monoclonal antibody as described previously (Wensvoort *et al.* 1986; Barman *et al.* 2012). The CSFV titers in 50% endpoint tissue culture infectious dose (TCID50) units/milliliter were determined on monolayers of PK-15 cells using the Reed and Muench method (Hierholzer and Killington 1996). The CSFV Indian classical strain, Assam strain, and local strain having a titer of $10^{3.25}$, $10^{2.00}$, and $10^{2.80}$ $TCID_{50}$/ml, respectively, were used for their first passage in PK-15 cells. Viral RNA was extracted from supernatants of infected cells by TRIzol (Invitrogen) using a standard protocol. Further, cDNA was prepared using a gene-specific reverse primer designed on the basis of the whole genome sequence of the CSF strain: E2 rev (5′-CCG CTC GAG ACC AGC GGC GAG TTG-3′) followed by PCR using E2 rev and E2 for (5′- CCC AAG CTT CGG CTA GCC TGC AAG G-3′) primer (Mayer *et al.* 2003). The PCR-amplified products were sequenced directly using a BigDye

terminator v 3.1 matrix standard kit and 3130xl Genetic Analyzer data collection software v3.0 (Applied Biosystems Inc). The E2 gene was sequenced at least three times from three independent RNA preparations to ensure a consensus sequence. Phylogenetic analysis of the E2 gene sequence of all the three strains with available GenBank sequences was performed by the Molecular Evolutionary Genetics Analysis software (MEGA4) using the maximum parsimony method. The robustness of the groupings in the neighbor-joining analysis was assessed with the 1000 bootstrap value (Kumar *et al.* 2008). The secondary structure of the putative E2 protein sequences was analyzed by PSIPRED software (http://bioinf.cs.ucl.ac.uk/psipred/).

The infection of CSFV vaccine strains did not show any cytopathic effect in PK-15 cells up to 15 passages. However, the CSFV strains showed positive IF and IP staining using the WH303 monoclonal antibody (Fig. 1). The CSFV Indian classical strain, Assam strain, and local strain showed a titer of $10^{7.25}$, $10^{5.00}$, and $10^{6.00}$ $TCID_{50}$/ml, respectively, after 15 passages. In addition, the replication of CSFV after each passage was confirmed by RT-PCR analysis using E2 gene-specific primers for the respective strains. The E2 gene sequences of all three vaccine strains following 15 passages were analyzed for their putative amino acid sequences. The nucleotide sequence of the E2 gene of the PK-15-adapted Indian classical strain showed 93.6% identity with its parental strain. Similarly, the nucleotide sequence of the E2 gene of the local strain and Assam strain showed 99.8% and 99.9% identity, respectively, with their parental strain after its adaptation in PK-15 cells (Table 1). The putative amino acid sequence of the E2 protein of the Indian classical strain and local strain of CSFV showed 95.6% and 99.4% identity, respectively, with the parental strain after its passage in PK-15 cells (Table 1). The CSFV strain Assam does not show any difference in amino acid level after its subsequent passage in PK-15 cells (Fig. 2). The local lapinized vaccine showed the mutation of N181K and W182L following its adaptation in PK-15 cells. A comparison of the amino acid sequences between the Indian classical strain and the reference sequence of the E2 protein of CSFV (NC_002657) showed differences of K720E, V744I, T886A, I971T, and A975E (Fig. 2). The conserved motif ^{829}TAVSPTTLR^{837} in domain A was found identical in all the strains independent of its number of passages in PK-15

Table 1 The nucleotide sequence and amino acid identity of the E2 protein gene of different strains of classical swine fever virus

	Indian classical	Indian classicalp	Local	Localp	Assam	Assamp
	Nucleic acid					
Indian classical		93.6	93.8	94.0	93.8	93.7
Indian classicalp	95.6		99.4	99.6	95.1	95.0
Local	95.0	99.4		99.8	95.3	95.2
Localp	95.6	100	99.4		95.5	95.4
Assam	93.3	94.4	93.9	94.4		99.9
Assamp	93.3	94.4	93.9	94.4	100	
	Amino acids					

p passaged virus

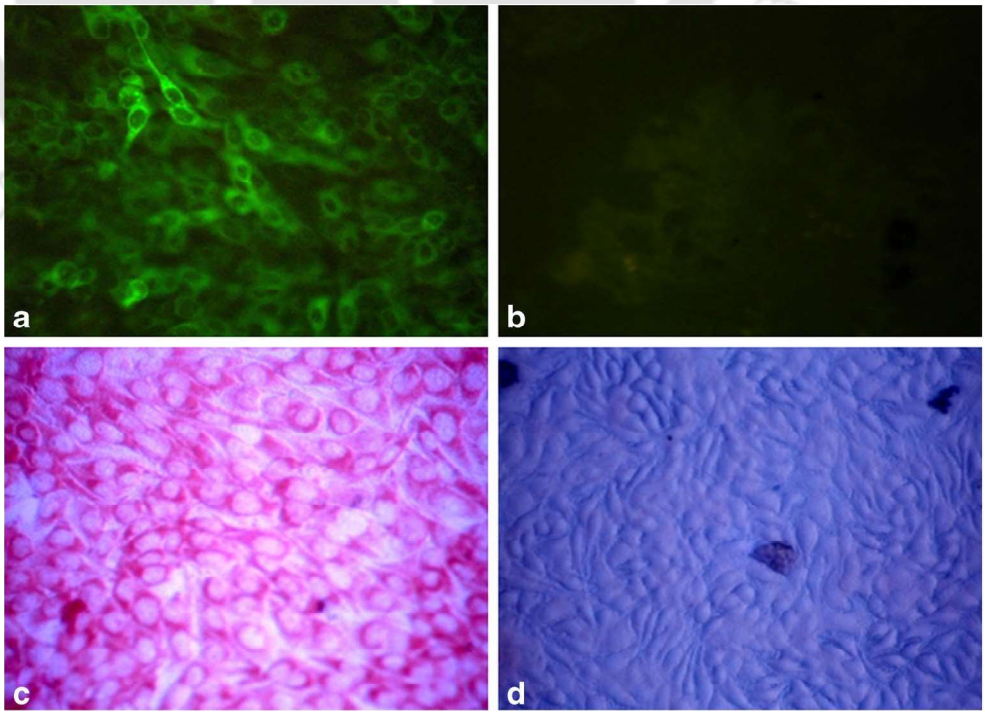

Figure 1 Immunofluorescence and immunoperoxidase staining of the classical Indian strain of classical swine fever virus following its infection in PK-15 cells. The infected cells showing positive fluorescence when stained with the WH303 monoclonal antibody followed by the fluorescein isothiocyanate conjugate secondary antibody and examined by fluorescence microscopy (**a**). No fluorescence was observed in uninfected cells (**b**). Similarly, positive peroxidase staining was observed when horseradish peroxidase-conjugated secondary antibodies were used to stain the infected cell (**c**). No peroxidase staining was observed in uninfected cells (**d**).

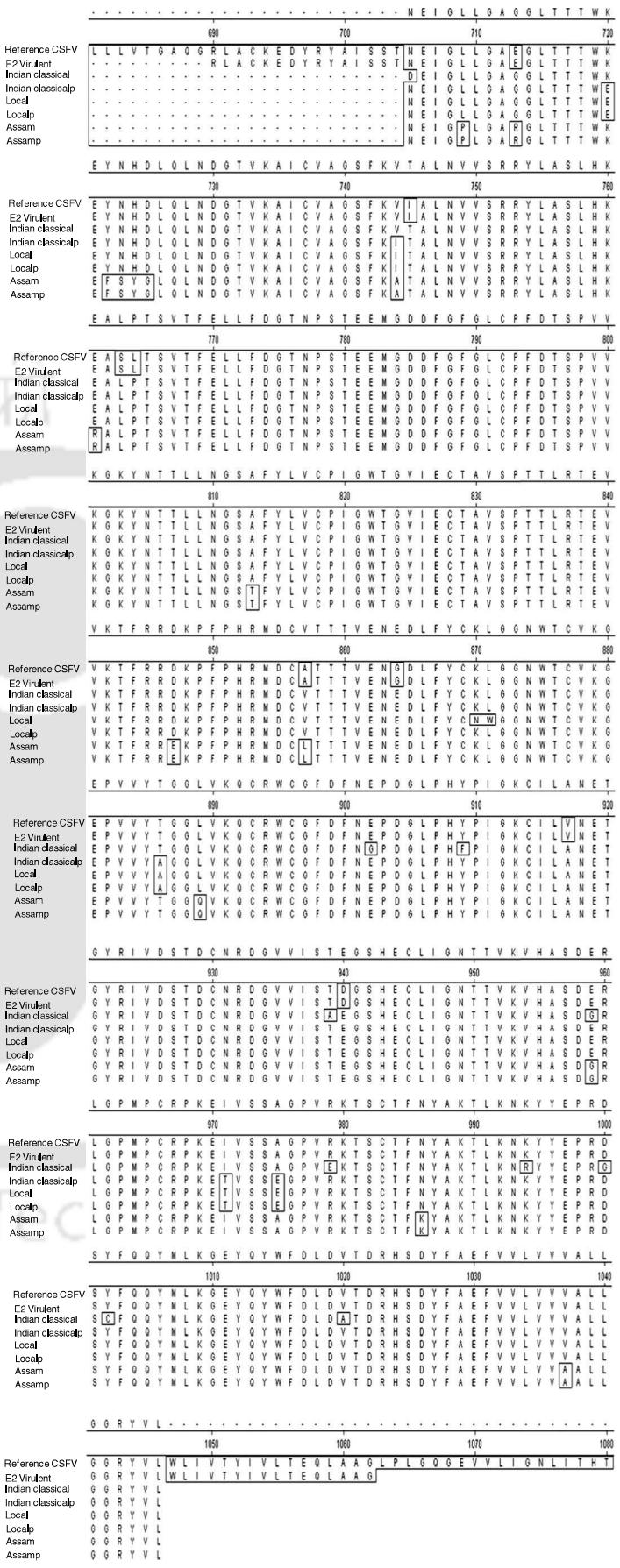

Figure 2 Amino acid sequence comparison of classical swine fever virus (CSFV) vaccine strains following its passage in porcine kidney cells (*p* indicates passaged virus). Amino acid sequence alignment was performed by ClustalW multiple alignment algorithm of the MegAlign program of the DNASTAR Lasergene 7 software package. GenBank accession number NC_002657 has been taken as reference sequence for CSFV. The E2 protein sequence encoded by the reference strain was listed as "E2 virulent" for the alignment in order to depict the correct amino acid position with respect to the CSFV polyprotein.

cells (Lin *et al.* 2000). Phylogenetic analysis of the E2 protein of cell culture-adapted vaccine strains with different CSFV strains showed clustering of the Indian classical strain with the local lapinized vaccine (data not shown). The amino acids ^{745}I, ^{763}S, ^{764}L, ^{864}G, ^{917}V, and ^{940}D were found unique to the virulent strain and were absent from the vaccine strains (Fig. 2). Our result showed that these amino acids were unchanged even after 15 passages suggesting its specificity for the virulent CSFV strains. How these specific changes contribute to the altered phenotype observed for the CSFV E2 protein gene remains to be determined. However, it has been shown recently that two single amino acid changes, S763L and P968H, in the E2 protein can cause the attenuation of CSFV in swine (Fahnoe *et al.* 2014). In addition, it has also been shown that T830A substitution in the E2 protein of CSFV can increase the pathogenicity (Tamura *et al.* 2012). Secondary structure analysis of the E2 protein of the PK-15-adapted Indian classical, local, and Assam strains showed the helix towards its carboxy terminus while the amino terminus is dominated by strands and coils similar to their non-passaged forms.

Our results of the cell culture adaptation of CSFV corroborate the earlier report where it has been shown that structural proteins can mutate faster than other viral proteins in order to selectively escape the host immune system (Wensvoort *et al.* 1990; Kwang *et al.* 1992). Moreover, the adaptation of CSFV in PK-15 showed subsequent high infectivity at a lower $TCID_{50}$ titer suggesting its better binding efficiency with the host cell surface receptor. A monoclonal antibody used in the study is targeted to the conserved amino acid sequences ^{829}TAVSPTTLR^{837} and reacts equally with all three strains of CSFV. Our finding corroborates the earlier report where a change in amino acid sequences in the E2 protein contributed to the better binding efficiency of CSFV to the cell surface receptor (Liang *et al.* 2003). The conserved amino acid sequences ^{829}TAVSPTTLR^{837} in all the CSFV strains as suggested earlier as linear epitope might not be important in the adaptation of the virus in vitro (Lin *et al.* 2000; Qi *et al.* 2008). The E2 protein is supposed to be a major determinant of virulence in CSFV, and changes in some critical amino acids might modulate its virulence (Risatti *et al.* 2005; Tamura *et al.* 2012). The existence of a conserved amino acid sequence in all three strains and their reactivity towards the WH303 monoclonal antibody suggest the possession of the immunogenic epitope in all three CSFV vaccine strains. However, the evaluation of cross-neutralization capacity in primary host sera with other vaccine strains and currently circulating wild strains can give the status of full protection in vaccinated animals. The study will be useful to understand the biology of CSFV and its repeated outbreaks in the vaccinated flocks. We have limited information about the biology of CSFV, and there are strains out there that can behave differently than what is taken as dogma. Further, exploring the mutations in the E2 protein gene of CSFV by reverse genetics will help us to modulate or attenuate the virus for the development of a better live recombinant vaccine.

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