CO-EXPRESSION OF CAPSID PROTEINS (P1) WITH OPTIMIZED LEVELS OF $3C^{PRO}$ OF FMD VIRUS IN INSECT CELLS USING BACULOVIRUS SYSTEM

Thesis

Submitted to the
DEEMED UNIVERSITY
Indian Veterinary Research Institute
Izatnagar – 243 122 (U.P.), India

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Doctor of Philosophy
(Veterinary Virology)

May, 2015

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled **"Co-expression of capsid proteins (P1) with optimized levels of $3C^{pro}$ of FMD virus in insect cells using baculovirus system"**, submitted by **V. Mouttou Vivek Srinivas**, Roll No. **1504**, for the award of **Doctor of Philosophy** in **Veterinary Virology** at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that **V. Mouttou Vivek Srinivas**, Roll No. **1504**, has worked for more than 39 months in the Institute and has put in more than 800 days attendance under me from the date of registration for the **Doctor of Philosophy** in this Deemed University, as required under the relevant ordinance.

(Madhusudan Hosamani)
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CERTIFICATE

We the undersigned members of Advisory Committee of, **Roll No. 1504**, a candidate for the degree of **Doctor of Philosophy** with the major discipline in **Veterinary Virology** agree that the thesis entitled **"Co-expression of capsid proteins (P1) with optimized levels of $3C^{pro}$ of FMD virus in insect cells using baculovirus system"**, may be submitted in partial fulfillment of the requirement for the degree.

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Doctor of Philosophy** of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Doctor of Philosophy** of the Deemed University, Indian Veterinary Research Institute, Izatnagar.

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Acknowledgment

Mere words can never suffice and express my deep sense of gratitude to several concrete and abstract hands that helped me in progressing forward and edified me from time to time. I shall always remain thankful to them all. Foremost, I would like to express my sincere gratitude to my advisor **Dr. Madhusudan Hosamani,** Senior Scientist, for his guidance and support throughout the course of this study.

A very special thanks to **Dr. Suresh H. Basagoudanavar,** without whose whole hearted support and inspiration, this thesis would have never seen the light of day.

My sincere thanks are due to **Dr. R. Venkataramanan,** the Joint Director, IVRI Bangalore for providing generous support, guidance and necessary facilities for this study.

I am extremely thankful to members of advisory committee; **Dr. B. P. Sreenivasa, Dr. P. Saravanan, Dr. R. P. Tamil Selvan, Dr. H. J. Dechamma** for their endeavors and morale boosting guidance during the period of my experiments. I am equally thankful to the SRFs **Varsha, Pravina, Dhanya, Deepthi** and Scientists of **FMD Research lab and QC/QA lab** for their help during my research. I am also thankful to **Dr. D. P. Singh** for translating mini abstract of thesis.

I would like to thank **Mr. Anthuraj, Mr. Ganapathy**. **Mr. Rajanna, Mr. Narayanappa, Mr. Nagan, Mr. Srinivasa** and **Mrs. Dhanalakshmi** for their technical support and loving nature.

I wish to express my whole hearted thanks to my batchmates **Vijayakumar, Sumanth, Asok, Latchumikanthan, Ilayabharathi, Barathidasan, Murugavel, Pavankumar, Rajagunalan, D.T. Karthick, Vijay Nagaraj** and my seniors **Dr. K. Karthik, Dr. Barathiraja, Dr. Kanthraj sir** who were my strength whenever I was struggling with my work at IVRI.

I thank my colleagues **Parveen kumar, Sonalika Mahajan, Raja Wasim, Ramesh Kumar, Bibhu Ranjan Dash, Sandeep Dash** and **Molalegne Bitew** for a pleasant and memorable company during our course work.

I owe my special thanks to my juniors **Munneshwaran, Sujit, Manoj, Ilayaraja, Mego, Maheshwary, Arun** and **Senthil** for their timely help and cooperation during my research.

My deepest gratitude goes to my family for their unflagging love and support throughout my life; this dissertation is simply impossible without them.

Words are inadequate to express my eternal gratitude and inexpressible sense of indebtedness towards my mother **Marisvary**, father **Mouttou Visvalingam**, my brother **Vijay Srinivas** and my fiancée **Nisha** for their encouragement, support and sacrifices in bringing me to this stage.

Finally, I express my sympathy to innocent **Guinea Pigs** sacrificed for the selfish cause of mine.

Vivek

V. M. Vivek Srinivas

Abbreviations

AcGFP : *Aequorea coerulescens* green fluorescent protein
BEI : Binary-ethyleneimine
BHK-21 : Baby Hamster Kidney cell line-21
BSA : Bovine serum albumin
$GPID_{50}$: 50% Guinea pig infective dose
bp : Base pair
cDNA : Complementary deoxyribonucleic acid
CPE : Cytopathic Effect
CsCl : Cesium chloride
DAB : Diaminobenzidine
DNA : Deoxy ribonucleic acid
dNTP : Deoxynucleotide triphosphates
DMEM : Dulbecco’s Modified Eagles Medium
EDTA : Ethylene diamine tetracetic acid.
ELISA : Enzyme Linked Immunosorbent Assay
EMCV : Encephalomyocarditis virus
et al. : and other people
FCS : Fetal Calf Serum
Fig. : Figure
FMD : Foot and Mouth Disease
FMDV Foot and Mouth Disease Virus
g : Grams
HRPO : Horseradish Peroxide
IRES : Internal ribosome entry site
IM : Intramuscular
IU : International Unit
LB : Luria Bertani
Log : Logarithm
kb : Kilobase pairs
kDa : Kilo Dalton

MCS	: Multiple cloning site
MEM	: Modified eagles media (Glasgow)
MP	: Monolayer passage virus
mRNA	: Messenger ribonucleic acid
Mut	: Mutant
OD	: Optical Density
OPD	: Orthophenylenediamine dihydrochloride
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate Buffered Saline
PCR	: Polymerase chain reaction
PD$_{50}$	: 50% protective dose
P10	: P10 promoter
Ph	: Polyhedrin promoter
Pro	: Protease
rec	: Recombinant
RE	: Restriction Enzyme
RBC	: Red blood cells
RT-PCR	: Reverse transcriptase PCR
SDS	: Sodium dodecyl sulphate
Sf-21	: Spodoptera frugiperda-21
SNT	: Serum neutralization test
TCID$_{50}$	: Tissue Culture Infective Dose $_{50}$
TE	: Tris EDTA
Tn-5	: Trichoplusia ni-5
TPB	: Tryptose Phosphate Broth
TVG	: Trypsin Versene Glucose
U	: Units
VNT	: Virus neutralization test

Units

^{0}C : Degree Centigrade

µl : Microliter

µg : Microgram

ng : Nanogram

ml : Milli-liter

mM : Millimolar

pM : Picomolar

rpm : revolution per minute

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Chapter 1

Introduction

Livestock sector which is an important sub-sector of Indian Agriculture, plays an important role in Indian economy. The overall share of livestock sector to the agricultural GDP is 23.7% and to the national GDP is 3.6% (National Account Statistics 2012, Central Statistical Organization, Ministry of Statistics and Programme Implementation). A number of infectious diseases continue to pose a major threat to animal health, impacting the growth of livestock sector. Among major livestock diseases, foot and mouth disease (FMD) is considered to be economically the most important one. It is a highly contagious viral disease of livestock causing severe vesicular disease mainly affecting cattle, buffalo, sheep, goat, pigs and other cloven-hoofed livestock and wild animals (e.g. antelopes, elephants, yaks, mithuns, blackbuck and ungulates etc) (Brooksby, 1982). As the disease is endemic in most of the regions of Asia, Africa and South America, it acts as major impediment in the free trade of animal and animal products between the developing economies and the lucrative global markets of the developed countries that are free from FMD. In India, direct annual loss due to FMD has been estimated to be more than 20,000 crores (Venkataramanan *et al.*, 2006).

The etiological agent, foot-and-mouth disease virus (FMDV), is classified within the Aphthovirus genus as a member of the Picornaviridae family (9th ICTV classification). The virus consists of non-enveloped particles that contain a positive-sense, single-stranded RNA of approximately 8.5 kb genome size. Its translation yields a polyprotein that is subsequently processed by virus-encoded proteases to produce the structural and non-structural proteins required for virus assembly and replication. In the mature virus, the genome is encapsidated in an icosahedral structure that is composed of 60 copies of four proteins (VP4, VP2, VP3, VP1). Viral 95 kDa polyprotein (P1) is cleaved by the viral 3C protease to yield VP0, VP1 and VP3 which self assemble to form the capsid (Fig. 1). Auto-catalytic cleavage of VP0 into VP2 and VP4 occurs during encapsidation of the viral genome to produce the mature virus (Curry *et al.*, 1997)

Seven serotypes (A, O, C, Asia 1, and South African Territories 1, 2, and 3) of FMDV have been identified serologically, and multiple subtypes occur within each serotype (Domingo *et al.*, 2003; Knowles and Samuel, 2003), and within each subtype the strains show a considerable degree of antigenic diversity and they often co-circulate in a particular geographical location (Brooksby, 1982; Mateu *et al.*, 1988). Among the seven known serotypes of FMDV, only three viz., O, A and Asia1 are prevalent in India. In India, serotype O is responsible for 80% of the confirmed outbreaks/cases, whereas Asia1 and A account for 12% and 8%, of the outbreaks, respectively (Subramanian *et al.*, 2013). Among all serotypes, type O is the most widely prevalent serotype in the world and is the major serotype isolated from recent outbreaks in many disease free countries (Cottam *et al.*, 2006). Type A is the most antigenically diverse of the Eurasian serotypes (Ansell *et al.*, 1994; Tosh *et al.*, 2002). The virus serotype Asia1 is endemic to Southern Asia and is now considered to be the least diverse type with a single subtype (Ansell *et al.*, 1994). The disease outbreak due to the type C has not been recorded in India since 1995 (Anon., 2007). According to the OIE/FAO World Reference Laboratory for FMD, the last type C incidence was reported in Brazil during 2004. SAT 1–3 serotypes have a natural reservoir in the African buffalo (*Syncerus caffer*) (Condy *et al.*, 1985) and are also characterised by their large degree of antigenic diversity (Sangare *et al.*, 2001).

FMD is controlled by ‘stamping out’ in the event of outbreaks in non-endemic countries and by vaccination programmes in endemic countries. The uncontrolled movement of animals, a low vaccination coverage and antigenic variation between serotypes are factors that cause problems in controlling FMD outbreaks (Azad *et al.*, 1995). In India, control measures such as slaughter policy would be impracticable and movement control is difficult to enforce. Therefore vaccination is considered as the only suitable control measures (Doel, 2003).

Currently, to control the disease, tissue culture-adapted field strains are used to produce serotype specific inactivated FMD vaccines. This requires cost intensive high-containment facility, which however poses the risk of virus escape from such facility. Sometimes there is possibility of incomplete virus inactivation leading to disease outbreaks in field. To address these concerns, research is ongoing worldwide to develop recombinant vaccines for FMD that are safe to produce, yet effective at eliciting protective immunity in animals. In this context, expression from the baculovirus system offers the potential for large-scale production of non-infectious FMDV capsids. Virus-like particles (VLPs), a result of recombinant DNA technology, are formed by viral structural proteins that can inherently self-assemble and mimic the morphology of the virus, without being infective or replicating (Brun *et al.,* 2011). In this context, expression from the baculovirus/insect cell system offers the potential for large-scale production of non-infectious FMDV capsids.

FMDV subunit vaccine based on empty capsid-like particles has been developed as one of the most promising alternatives to conventional vaccines (Li *et al.,* 2008). These recombinant non-infectious FMDV empty capsid-like particles are potentially useful for the development of diagnostic techniques (Basagoudanavar *et al.,* 2013) and vaccines (Lewis *et al.,* 1991). Empty capsids of FMDV are as antigenic and immunogenic as authentic FMDV, but produce no infection, because they have no RNA genome. The VLPs present conformational epitopes more similar to those of native viruses, with efficiency to generate humoral and cell-mediated immune responses (Kushnir *et al.,* 2012). The assembly of FMDV capsid in infected cells requires the cleavage of P1 polyprotein by non-structural protein 3C into individual structural proteins. One of the initial polypeptide cleavages, mediated by the 2A protein, is a co-translational cleavage at the N-terminus of the 2B protein (De Felipe *et al*. 2003). Each capsid subunit is formed upon the assembly of one copy of VP0, VP1, and VP3, and VP0 is the precursor of VP2 and VP4. VP1, VP3, and VP0 spontaneously form the 5S protomer, which subsequently assembles into a 14S pentameric subunit that, in turn, assembles into the icosahedral procapsid (Fig. 1). Encapsidation of viral RNA to produce mature virions is accompanied by the cleavage of VP0 to VP2 and VP4 which is not mediated by 3C and mechanism is not known completely (Curry *et al*., 1997).

VLPs have been generated through the co-expression and self-assembly of their components in yeasts (Freivalds *et al.,* 2011), Escherichia coli (Yin *et al.,* 2010), mammalian cells (Wu *et al.,* 2010) and insect cells (Baek *et al.,* 2011). As an essential eukaryotic expression system modifying and processing the proteins expressed in insect cells, using baculovirus-based expression system (BES) plays a key role in self-assembly and release of VLPs (McClenahan *et al.,* 2010). The only licensed and commercially available VLP based animal vaccine is porcine circovirus type 2 (PCV2) VLP-based vaccine Porcilis PCV® (Intervet International, Netherland). Co-expression of capsid precursor protein, P1-2A and non-structural protein, 3C results in the formation of empty virus like particles. The non-structural protein 3C is a thiol-protease that is responsible for most of the cleavage events undergone by the viral polyprotein. So far, attempts to produce capsid vaccines are not highly successful due to lower yields of coat proteins, a feature that is strongly attributed to toxicity associated with 3C protease essential for processing of polyprotein. This may result from the fact that the FMDV 3Cpro has a number of cellular targets including certain translation initiation factors (eIF4G and eIF4A) (Belsham, 2000), cytoskeleton components (Armer *et al.*, 2008) and histone H3 (Falk *et al.*, 1990). The 3C protease induces the proteolytic processing of histone H3, which is probably linked to the inhibition of host transcription observed in infected cells. There has been some success in using different systems for the production of the FMDV empty capsid particles. Hence moderating its protease activity through point mutations (Sweeney *et al.*, 2007) or reducing the expression of 3C protease (Chung *et al.*, 2010; Polacek *et al.*, 2013; Porta *et al.*, 2013b; Gullberg *et al.*, 2013) are some of the strategies used to improve the yields of capsid proteins. This is achieved through reducing the expression of 3C molecules by introduction of IRES or frame shift signal sequence upstream to 3C gene. Thus it is critical to obtain optimized expression of 3Cpro for efficient P1 structural protein processing.

Based on these observations, the present study was proposed to express the capsid proteins (P1-2A) along with reduced 3C protease activity to improve the yields of processed capsids by two strategies. These include, using FMDV-IRES sequences upstream of 3C to drive bi-cistronic expression of capsid sequences or, through expressing capsid proteins and 3C driven by strong polyhedrin promoter and relatively weaker P10 promoter, respectively. Such studies first to be attempted in insect cells expected to contribute in improving the yields of empty capsid of FMDV, which could be potentially applied for developing newer diagnostics tools and/ or vaccines with the following objectives.

1. To generate recombinant baculoviruses expressing capsid proteins along with reduced levels of 3Cpro for FMDV serotype O virus (IND-R2/75).
2. To characterize the recombinant baculoviruses expressing FMDV capsid protein and evaluating their immunoreactivity.
3. To evaluate the immunogenicity of the recombinant proteins in guinea pigs.

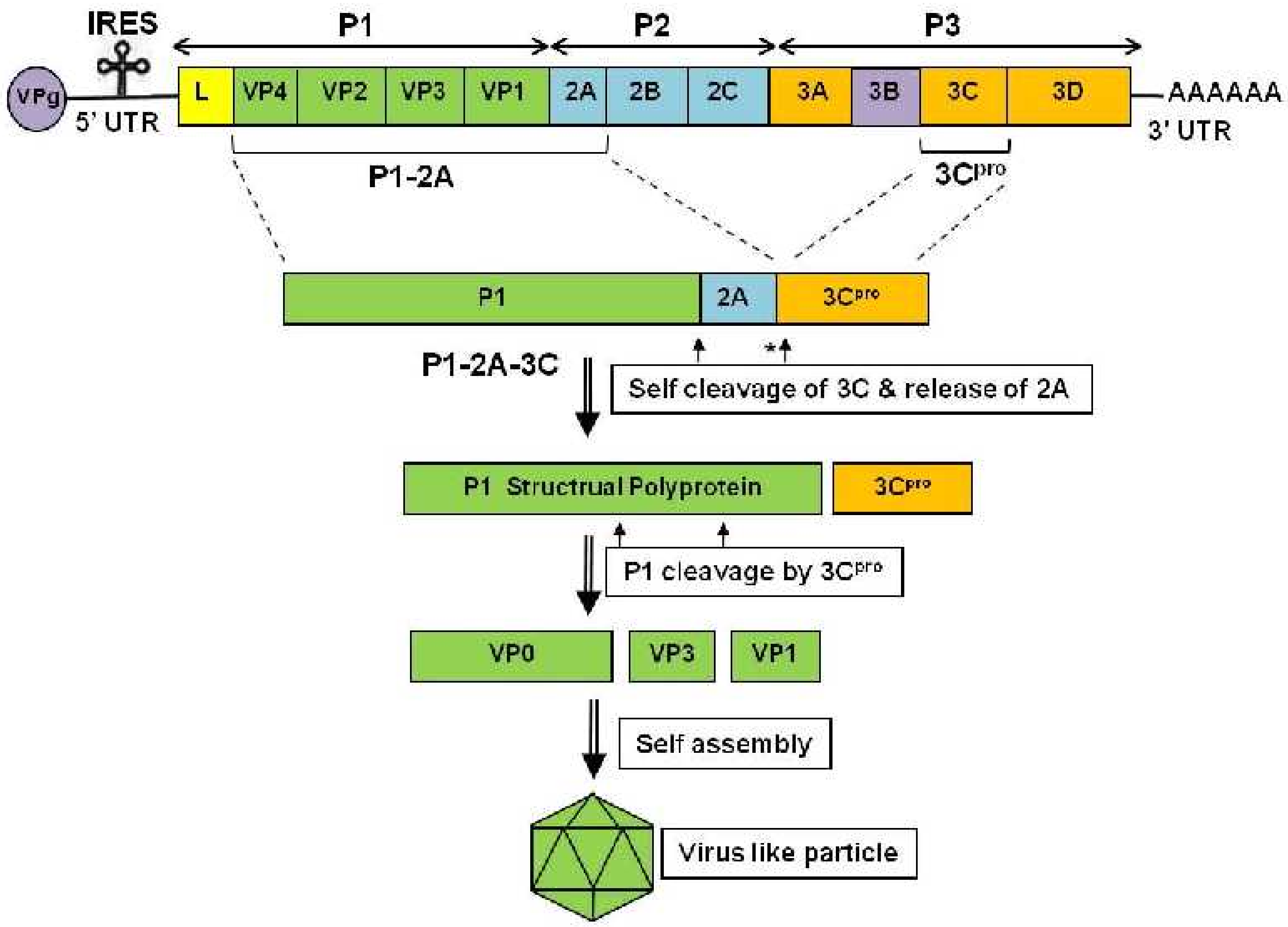


Fig. 1. Schematic representation of recombinant FMD virus like particle synthesis. Proteolytic cleavage of expressed FMDV P1-2A-3C polyprotein subsequently into VP0, VP3 and VP1 structural proteins would allow their self assembly into empty virus like particles. In P1-2A-3C, targets of 3C are indicated by upright arrow while asterisk (*) indicates self cleavage site of 2A.

Chapter 2

Review of literature

FMD is a major economically important livestock disease, which continues to be a global problem in international trade with wide range of impacts on livestock production and economic development of agriculture-based country. FMD is major constraint to international trade of animals and animal products (Leforban, 1999), hence the disease has been listed as ‘notifiable disease’ of the OIE. The disease causes very high economic losses, due to trade embargoes besides crippled agricultural draught power in developing countries like India, combined with direct losses often associated with high calf mortality, prolonged convalescent, carrier status, low milk production. This has an enormous impact on the Indian economy with the direct annual loss roughly estimated at US $ 800 million while indirect losses in market are much more (Bandyopadhyay, 2003). FMD is one of the most highly contagious diseases of animals, as FMDV rapidly replicates and spreads within the infected animal and among in-contact susceptible animals by aerosol. Disease signs can appear within 2 to 3 days after exposure and can last for 7 to 10 days.

FMDV is among the first animal virus discovered by mankind, and there is huge accumulation of knowledge on the virus. Yet there are many aspects of this virus which are unclear due to diverse nature of the FMDV, its epidemiology and transmissibility, and as a result, deficiency of the currently available diagnostic and vaccines and/ or logistic support required to eliminate this virus. Following sections cover the FMD virus, its biology and current vaccine approaches, that are relevant to the scope of the work proposed.

2.1. Foot and mouth disease virus (FMDV)

The first written description of FMD probably occurred in 1514, when Fracastorius described a similar disease of cattle in Italy (Fracastorius, 1546). Almost 400 years later, Loeffler and Frosch, (1897) demonstrated that a filterable agent caused FMD. This was the first demonstration that a disease of animals was caused by a filterable agent and ushered in the era of virology. Subsequently, it was shown that the agent, FMDV consists of a single-stranded, plus-sense RNA genome of approximately 8,500 bases surrounded by four structural proteins to form an icosahedral capsid (Rueckert, 1996). FMDV is the type-species of the *Aphthovirus* genus of the *Picornaviridae* family. The only other member of this genus is equine rhinitis A virus (King *et al.,* 2000). Seven serotypes (A, O, C, Asia 1, and South African Territories 1, 2, and 3) have been identified serologically, and multiple subtypes occur within each serotype (Bachrach, 1968).

The virion is non-enveloped with a pseudo T=3 icosahedrons of 25nm in diameter (Bachrach,1968) with molecular weight of 8.3 to 8.9x 10^6 daltons and its buoyant density in CsCl is 1.43-1.45 g/cm^3 . The intact purified virus particle has a sedimentation coefficient of 146s. The virion consisting of a single-stranded RNA genome and 60 copies each of four structural proteins (1A [VP4], 1B [VP2], 1C [VP3], and 1D [VP1]). The FMDV genome has a basic organization similar to those of other members of the *Picornaviridae*, and the nomenclature for the viral proteins was established by Rueckert and Wimmer (1984). The characteristic feature of FMDV, like other virus, is its antigenic and genetic diversity, which has been reflected by the presence of seven distinct serotypes and constant emergence of variants within each serotype (Domingo *et al.,* 2003; Knowles and Samuel, 2003). Upon virus entry into a cell, the viral genome is rapidly translated into a polyprotein which is co- and post-translationally cleaved by viral proteinases into several partially cleaved, likely functional, intermediates and ultimately into 12 mature proteins (Rueckert, 1996).

2.2. Genome Organization

The FMDV genome has a basic organization similar to those of other members of the *Picornaviridae,* and the nomenclature for the viral proteins was established by Rueckert and Wimmer (1984). The genome of the FMDV consists of a large single open reading frame (ORF) flanked by highly structured 5’ and 3’untranslated regions (5’UTR and 3’UTR), respectively.

2.2.1. 5’ UTR of FMDV

The 5’ UTR of FMDV contains about 1,300 bases (Grubman *et al*., 1984) and can be divided into five functional elements, which play roles in virus translation and RNA replication. The most 5’ segment, the S fragment, encompasses about 360 bases and folds into a long stem-loop (Newton *et al.,* 1985). The function of the S fragment is not known, but analogies with other picornaviral genomes suggest that it may play a role in maintaining genome stability in infected cells (Barton *et al*., 2001) and may also be involved in the binding of proteins involved in genome replication (Xiang *et al*., 1995).

Following the S fragment, there is a poly(C) tract comprising over 90% C residues with a small number of U and A residues. This segment is over 100 bases in length; however, the length of the poly(C) tract can be extremely variable (Costa Giomi *et al*., 1984). Although an early study suggested that the length of the poly(C) tract was associated with virulence (Harris and Brown**,** 1977), other studies have been unable to correlate poly(C) length with this property of the virus (Costa Giomi *et al*., 1984). Just downstream of the poly(C) tract, there is a series of RNA pseudoknot structures of unknown function (Escarmis *et al*., 1995).

Downstream of the pseudoknots, there is a short hairpin loop structure, the *cis*-acting replicative element (*cre*). It has a stem-loop with a conserved AAACA sequence in the loop region. The *cre* is essential for RNA genome replication. In contrast to the case for other picornaviruses, where the *cre* is located within different regions of the ORF, the *cre* of FMDV is located within the 5' UTR (Mason *et al*., 2002).

2.2.1.1. IRES Element and its function

The region between the *cre* and ORF contains a series of highly conserved stem-loop structures, which together constitute the internal ribosome entry site (IRES). Translation of FMDV RNA is initiated internally, under the control of IRES sequence (Belsham and Brangwyn, 1990). Initiation of translation mediated by IRES elements represents an alternative to the cap-dependent translation initiation mechanism used for most cellular mRNAs. The 5′ end of all cytoplasmic eukaryotic mRNAs has a cap structure (m7GpppN…) which plays a crucial role during translation initiation recognized by the initiation factor eIF4F (heterotrimer) comprises the translation initiation factors eIF4E (that binds to the cap), eIF4A (an RNA helicase) and eIF4G. It is generally believed that the small ribosomal subunit interacts with the eIF4F complex (bound at the 5′ cap) and then migrates with it along the 5′ UTR in a 5′ to 3′ direction. The presence of eIF4A is believed to facilitate the unwinding of RNA secondary structure in IRES. This migration is called scanning and continues until an AUG codon (Kozak, 1989) is encountered. At this point, the ribosome pauses, the large ribosomal subunit joins and polypeptide synthesis will commence. The ability of the scanning process to recognize the correct initiation codon can be inhibited by complex RNA structure and by the presence of additional AUG codons. In contrast, internal initiation of translation mediated by IRES involves the direct recruitment of the translational machinery to an internal position in the mRNA usually with the help of cellular *trans*-acting factors. IRES elements were first identified within picornavirus RNAs (Pelletier and Sonenberg, 1998). Consistent with their role in picornavirus translation, IRES-dependent translation initiation can bypass stress conditions that are inhibitory for cap-dependent translation initiation (Martínez-Salas *et al.*, 2001). Such stress conditions occur following eIF4G cleavage that is induced during picornavirus infection as a consequence of the action of viral proteases Lb (FMDV) or 2A (poliovirus).

The IRES element is believed to adopt a tertiary structure that is essential for internal initiation of translation. Two major groups of picornavirus IRES structures have been classified, first the entero-/rhinoviruses and secondly, the cardio-/aphthoviruses. Neither type of IRES has any apparent similarity to that of hepatitis C virus (HCV). The absence of apparent structural conservation between different IRES elements may reflect the diversity of strategies used by IRES elements to interact with the translational machinery.

2.2.1.2. Structural Motifs in FMDV IRES and their role

The first 85 nt contain domains 1 and 2. The first 21 nt of the domain 1 is now recognized as the right arm of the *cre* stem-loop (Mason *et al.*, 2002). However, the presence of these sequences upstream of the IRES may help to stabilize the structure of domain 2 that is essential for IRES activity under certain conditions (Kuhn *et al.*, 1990). The 60 residues of domain 2 are predicted to form a highly conserved stem-loop structure that has a pyrimidine-rich sequence at its apical loop (UCUUU). This stem-loop contains the main binding site for a 57 kDa protein known as the polypyrimidine tract binding protein (PTB) (Luz and Beck, 1991). The apical part of domain 3 within the FMDV IRES contains stable stem-loop structures that are believed to form a four-way junction. Two phylogenetically conserved motifs, GNRA and RAAA, that are present in the apical loops, constitute essential regions for the activity of the IRES within cells (Robertson *et al.*, 1999). Recently, Gullberg *et al.* (2013) mutated the GNRA motif to GTTA and been successful in reducing the translation efficiency of their downstream cistron. The GNRA motif appears to be responsible for the organization of the adjacent stem-loops, as deduced from accessibility studies using ribonucleases and dimethyl sulphate. Binding of synthetic stem-loops bearing UCCG or GUAG sequences in place of the GNRA motif were significantly reduced in their binding efficiency to domain 3 compared to the wild-type GUAA hairpin (Fernández- Miragall and Martínez -Salas, 2003). Analysis of sequences located at the base of domain 3 strongly suggested that formation of a helical structure around positions 88 and 297 of the FMDV IRES is needed for efficient internal initiation of translation by contributing to the stability of the entire domain. If domain 3 plays an essential role in the organization of IRES architecture as suggested (Ramos and Martinez-Salas, 1999) then small changes in the stability of this domain may induce a reorganization of the whole element with important consequences for IRES function. The RNA-RNA interactions observed *in vitro* between separated domains of the FMDV IRES, in the absence of proteins, suggest that the IRES fragments fold in specific forms, depending upon environmental conditions (RNA concentration, ionic conditions and temperature). The central region (domain 3) of the FMDV IRES is unique in its ability to interact with each of the other domains suggests that domain 3 acts as a scaffold structure that holds together the remaining domains of the IRES, and secondly, that it is essential to determine intermolecular interactions with other IRES molecules. In the context of a picornavirus infection, physiological changes affecting ionic conditions, pH gradients, free radical formation, expression of specific RNA binding proteins, etc., may induce the reorganization of IRES structure that could change the tertiary structure of the IRES within infected cells and, as a consequence, translation efficiency may be modulated (Carrasco, 1995).

Sequence comparison between the FMDV and EMCV RNAs at the base of domain 4 is of great interest since there is both primary sequence and secondary structure conservation. Two A-rich internal bulges are conserved within the secondary structure of the different field isolates of FMDV as well as in the related EMCV IRES (Hellen and Wimmer, 1995). The eIF4G interacts with the domain 4 for bridging the small ribosomal subunit; So any single point substitution, deletion or insertion at this A-rich internal bulges severely impaired or abrogated the FMDV IRES activity in the context of dicistronic constructs (Martínez-Salas *et al.*, 2001).

2.2.2. 3' UTR of FMDV

The 3' UTR, which follows the ORF termination codon, contains a short stretch of

RNA which folds into a specific stem-loop structure (Pilipenko *et al*., 1992) of about 90 nt followed by a poly(A) tract of variable length carried on the genome (Dorsch-Hasler *et al*., 1975). The 3' UTR also appears to be important for genome replication (Rohll *et al*., 1995). The 3' UTR can bind a number of picornaviral proteins that are involved in RNA replication (Harris *et al*., 1994). Deletion or replacement of the FMDV 3' UTR reduced the efficiency of *in vitro* translation and blocked the ability to recover viable virus from transfected cells (Saiz *et al*., 2001). The poly (A) tract probably functions in FMDV translation (Lopez de Quinto *et al*., 2002) and may also play a role in picornavirus RNA replication (Herold and Andino, 2001). Unlike cellular mRNAs- where the poly (A) is added post-translationally - the poly (A) tract of the picornaviruses is encoded by the viral genome itself (Dorsch-Hasler *et al*., 1975).

2.2.3. Open reading frame (ORF) of FMDV

The ORF of the genome can be further classified, based on the initial cleavage products into four regions *viz.*, L, P1, P2, and P3. The genome is monocistronic and encodes a single polyprotein that undergoes steps of proteolytic cleavages to yield four structural proteins and eight non-structural proteins (Grubman *et al*., 1984).

The four Structural (capsid) proteins: 1A, 1B, 1C, and 1D (also known as VP4, VP2, VP3, and VP1, respectively) are encoded by the P1. N-terminal half of the ORF (L_{pro}), and with the exception of 1A, which is excluded from the virion surface, are involved in antigenicity and binding to a subset of RGD-dependent integrins and heparan sulfate proteoglycan receptors on the cell surface (Jackson *et al*., 2003).

Non-structural proteins represent about two-thirds of the ORF-encoded proteins and include Lpro encoded by the L gene; 2A, 2B, 2C encoded by P2, and 3A, 3B, 3Cpro, and 3Dpol encoded by the P3 region (Porter, 1993).

FMDV polyprotein processing is mediated by Lpro, 3Cpro, and 2A. Lpro is a papain-like protease that, in addition to excising itself from the polyprotein, cleaves the cellular translation initiation factor eIF4G, resulting in a shutoff of host cap-dependent translation (Lopez and Martinez, 2000). 3Cpro, a member of the trypsin family of serine proteinases, performs all but three of the cleavages leading to mature viral proteins and also cleaves host cell proteins (Vakharia *et al*., 1987). FMDV 2A mediates autocleavage at its C terminus, apparently by inducing a ribosomal skip during polyprotein synthesis (Donnelly *et al*., 2001). Although the functions of the FMDV 2B and 2C proteins are unknown, preliminary work suggests that, similar to those of other picornaviruses, they localize to endoplasmic reticulum (ER)-derived vesicles, the sites of viral genome replication and is responsible for virus-induced cytopathic effects (Tesar and Marquardt, 1989) and resistance to guanidine, an inhibitor of RNA replication, has been mapped to mutations in 2C. 3A is thought to be a multifunctional integral membrane protein that enhances viral RNA synthesis by 3Dpol and stimulates cleavage of the 3CD precursor (Polatnick, 1980). It is also associated with viral induced membrane vesicles, cytopathic effect and inhibition of protein synthesis (Xiang *et al.,* 1995). FMDV encodes three non-identical copies of genome-linked 3B; a protein covalently linked to the 5' end of the genome and is required for viral RNA replication and encapsidation of the picornaviral RNA (Falk *et al*., 1990). The 3D gene encodes the viral RNA-dependent RNA polymerase, and 3D and 3A co-localize with ER membrane-associated replication complexes (O'Donnell *et al*., 2001).

2.3. The virus structure and its properties

The FMD virion appears to be a round particle with a smooth surface and a diameter of about 25 nm by electron microscopy (Bachrach, 1968). The mature FMDV capsid is non-enveloped with a diameter of ~ 300°A consisting of 60 copies of each of four structural proteins. The structural proteins, VP1 to -3, fold into an eight-stranded wedge-shaped β barrel which fit together to form the majority of the capsid structure (Acharya *et al*., 1989). The VP4 protein is buried within the capsid and has a myristyl group covalently attached to its N terminus (Chow *et al*., 1987). The strands of the β barrels of VP1 to -3 are connected by loops which form the outer surface of the virion. Another feature of the virion is the presence of a channel at the fivefold axis which permits the entry of small molecules, such as CsCl, into the capsid, resulting in FMDV having the highest buoyant density of the picornaviruses (Jackson *et al.,* 2003).

Unlike those of other picornaviruses, the FMDV capsid is dissociated at pH of below 6.5 into 12S pentameric subunits (Brown and Cartwright, 1961). The reason for this instability is thought to be a cluster of His residues at the interface between VP2 and VP3 which become protonated at low pH, weakening the capsid through electrostatic repulsion

(Ellard *et al.*, 1999). This low-pH-induced instability of FMDV leads to differences in the mechanism of its uncoating upon infection of cells compared to that for other picornaviruses and also probably plays a role in the targeting of the virus to specific tissues and organs in susceptible hosts. The FMDV is distinguished from other picornaviruses by the lack of a surface canyon, or pit which has been shown to be the receptor binding site for the entero- and cardioviruses (Belnap *et al.*, 2000).

X-ray crystallographic studies of the FMDV showed that the capsid is covered by portions of VP1-3 proteins (Jackson *et al.*, 2003), all of which are having a core structure of a highly conserved wedge shaped eight stranded β barrel, composed of two four stranded β sheets. Conventionally, β strands of the two sheets are labelled alphabetically following the amino acid sequence, namely CHED and BIDG while loops joining these strands derive their nomenclature on the basis of the strands they join. The loops along with the C- termini portion of these three proteins are exposed on the virion surface where as N- termini are located interiorly. Hence, amino acid residues of these portions contribute immensely towards defining the antigenicity of the virion (Mateu, 1995).

2.4. Viral transcription and genome replication

Picornavirus RNA replication presents a number of unique challenges. The 5’ end of the genome RNA is covalently linked to VPg, and the 3’ end has a genetically coded poly(A) tail. Thus, the viral RNA-dependent RNA polymerase (3Dpol) must distinguish between viral RNAs and cellular mRNAs, which also contain 3’ terminal poly (A) tracts. In addition, since the mRNA and the genome RNA are the same molecule, with the exception of the genome-linked VPg, there must be a mechanism to distinguish RNAs which are bound for the ribosome and those which will be packaged into virion particles (Grubman and Baxt, 2004).

The first step in picornavirus RNA replication is the synthesis of a minus-strand RNA molecule. This system has not been studied in FMDV; however, the models of RNA replication developed for poliovirus are probably quite similar (Paul, 2002). It is thought that translation of the plus-strand RNA must cease before minus-strand synthesis begins (Gamarnik and Andino, 1998). The mechanism of this shutdown of translation of the plus strand is unclear; however, it has been proposed that, in poliovirus-infected cells, when the polymerase precursor (3CD) accumulates in the cell, it binds to the 5’ cloverleaf structure and modifies the affinity of PCBP for the IRES, an interaction which is essential for translation. Whether 3CD plays a similar role in FMDV RNA replication is not known; however, it has been shown that in FMDV-infected cells, this protein is rapidly cleaved to 3Cpro and 3Dpol (Grubman *et al*., 1984).

One model proposes that initiation of minus strand begins after circularization of the genome facilitated by the interactions of poly (A) binding protein (PABP) with the 3' poly(A) tail and the PCBP-3CD-5' cloverleaf structure (Herold and Andino, 2000). In the FMDV genome, the 5' interactions probably take place within the S fragment and may also involve the poly(C) tract. Since initiation of minus-strand synthesis occurs in the cytoplasm in the presence of cellular mRNAs, which also contain poly (A) tails, picornaviruses must have developed mechanisms enabling the polymerase to recognize viral RNA. In picornavirus-infected cells, both plus- and minus-strand RNAs are linked to VPg (Nomoto *et al*., 1977), and the presence of a small protein-linked dinucleotide, VPgpUpU, in infected cells (Crawford and Baltimore, 1983) suggested that VPg might be a primer for the RNA polymerase.

The *cre* provided a rational mechanism for both the uridylylation of VPg and the ability of the polymerase to discriminate viral RNA from cellular mRNAs. This conserved motif is required for poliovirus and rhinovirus minus-strand synthesis (McKnight and Lemon, 1998), and the first two A's serve as the template for the synthesis of VPgpU and VPgpUpU and for viral replication initiation in enteroviruses (Rieder *et al*., 2000). Mutations in this motif within the FMDV *cre* severely reduced viral replication in cell culture; however, the *cre* is positionally independent within picornavirus genomes (Yin *et al*., 2003). It is not clear at this time whether free VPg is utilized in the initiation step or whether a cleavage precursor (3AB, 3ABC, or 3BCD) is needed.

Following the initiation reaction, elongation of the minus strand begins, catalyzed by 3Dpol. For this to occur, the initiation complex must translocate to the 3' end of the plus-strand template. The mechanism by which this occurs is unknown, but one hypothesis suggests that binding of PABP to the poly(A) tract positions this region of the plus strand near the *cre* (Paul, 2002). The elongation of the nascent strands results in the formation of a double-stranded molecule, the replicative form (RF) (Wimmer *et al*., 1993). Free minus strands are not detectable *in vivo*.

After formation of the RF, new plus-strand synthesis can begin. The initiation of plus-strand synthesis from the RF has not been elucidated; however, two possible mechanisms to generate VPgpUp have been suggested. The first proposes either using existing uridylylated VPg, made in abundance during minus strand synthesis, or uridylylating VPg at the 3’ end of the minus strand (Paul, 2002). The second hypothesis, which also disputes the mechanism for the initiation of minus strands presented above, proposes that VPgpUpU is generated on the 3’ poly(A) tail of the plus strand and utilized for minus-strand synthesis, while *cre*-generated VPgpUpU is utilized for plus-strand synthesis (Murray and Barton, 2003). Since the data which led to the latter hypothesis was generated totally with cell-free systems, it is still uncertain whether these mechanisms are utilized in infected cells. In addition, it has recently been suggested that FMDV *cre* function can be complemented in *trans* (Tiley *et al*., 2003). While more studies are necessary to confirm this result.

For plus-strand synthesis to proceed, the RF must be unwound. The mechanism for this is also unclear. The picornavirus 2C protein both has ATPase activity and contains helicase motifs but helicase activity has not been demonstrated (Pfister and Wimmer, 1999). It has been shown that 2C and a cellular protein (p38) bind to the minus-strand 3’ stem-loop (Rohll *et al*., 1995), and this may act to destabilize the RF molecule. The possibility of involvement of either a cellular helicase or a nuclear protein has also been suggested, since the RF is infectious when transfected into whole cells but not when transfected into enucleated cells (Morgan-Detjen *et al.,* 1978). The elongation of the plus strand by 3Dpol also occurs by an unknown mechanism.

2.5. FMD Control measures: Vaccines and vaccinology

The vaccination with inactivated trivalent vaccine is considered as the only suitable control measure for endemic countries like India. There several limitations of the current vaccine, such as lack the ability to promote good cellular response, unforeseen risk of improper inactivation and virus release into the environment from the production facilities and narrow spectrum of immunogenicity. Another problem is that FMD virions are remarkably thermo labile, and moderate heat induces their dissociation into pentameric subunits in the vaccine, leading to dramatic losses of immunogenicity (Meloen *et al*., 1979). The currently used vaccines have short duration of immunity and interference of vaccine-induced immunity by maternal immunity. Other difficulties in using inactivated FMD vaccines include: high-containment facilities required for the production of vaccine; the vaccine does not induce rapid protection, there is a chance of susceptibility of vaccinated animals prior to the induction of the adaptive immune response and, vaccinated animals can become long-term carriers (Grubman and Baxt, 2004).

To address these limitations research has been going on for the production of more desirable vaccine formulations like empty viral capsids (virus like particles), live attenuated vaccines, live vectored vaccines and DNA vaccines etc. which are achieved through rDNA technology. With the development of information concerning viral capsid structure, researchers had determined that VP1, one of the FMDV capsid proteins, had a prominent surface exposure (Sobrino et al., 2001). Based on this information and related researches, a number of strategies were designed to develop subunit vaccines as alternatives to conventional inactivated vaccine. Bacharach *et al*. (1975) obtained VP1 isolated from purified viruses inducing a neutralizing antibody response in swine. Kupper *et al.,* (1981) cloned the VP1 gene, transferred it into E.coli in the form of recombinant plasmid and harvested the VP1. New subunit vaccines for FMDV are frequently reported, however, there are no subunit vaccines revealing advanced effective protection compared with traditional inactivated vaccines. In spite of it, subunit vaccines are still widely regarded as optimal candidates instead of classical inactivated vaccines for advantages, such as high security and the use of serological tests that can differentiate infected from vaccinated animals. This ideal can be most likely achieved by the development of empty capsid vaccines.

2.6. Virus-like Particles (VLPs)

Foot-and-mouth disease virus-like particles (VLPs) are non-replicating, non-pathogenic particles that have structural characteristics and antigenicity similar to the parental virus. They are similar in conformation to intact virions and are formed by the self-assembly of processed capsid proteins. A critical component of VLP experimental vaccines is the ability to process the viral capsid polyprotein (P1) into cleaved products that can then assemble into VLPs. There are different protein-processing paths that have been pursued by the inclusion of non-structural viral proteins 2A, 2B and 3C proteinase. There are several expression systems for the production of VLPs, including various mammalian cell lines, either transiently or stably transfected or transduced with viral expression vectors, the baculovirus/insect cell or larvae systems, various species of yeast (*Saccharomyces cerevisiae* and *Pichia pastori*), *E. coli* and other bacteria.

A yeast-derived VLP experimental FMD vaccine was initially described in 2003 (Balamurugan *et al.,* 2003). The capsid from a serotype O strain induced SN and ELISA titers in guinea pigs and these animals were protected against homologous challenge. The co-expression of either recombinant bovine IFN-γ, IL-18 or HSP-70 and VP1 (Shi *et al.,* 2007; Su *et al.,* 2007) constructs has been shown to enhance SN and cell-mediated immune responses in mice; however, no livestock vaccine efficacy studies have been reported.

Hepatitis B virus core (HBc) particles self-assemble into capsid particles and are extremely immunogenic. However, formation of VLPs can be restricted by size and structure of heterologous antigens. The first report of the use of the HBc system for expression of amino acids 141–160 of the VP1 protein of FMDV was made over 25 years ago, and the immunogenicity of the VLP structures was reportedly similar to that of intact FMD particles (Clarke *et al.,* 1987). The formation of VLP in mammalian cells by modified HBc fused with specified FMDV multi-epitopes was also studied. Complete VLP structures with one construct was confirmed by electron microscopy and induced both humoral (peptide- and FMDV-specific antibody) and cell-mediated immunity responses in mice (Zhang *et al.,* 2007)

2.7. Baculovirus Expression Vector System (BEVS) and BEVS derived FMD VLPs

The BEVS technology, developed by Max Summers, Gale Smith and their colleagues at Texas A&M University, has unique biological advantages over bacterial, yeast or mammalian protein expression systems. A major advantage is the quick turnaround time for the expression of recombinant proteins that show biological activity, antigenicity, and immunogenicity similar to authentic natural proteins. Also, the vectors are not dependent on helper viruses nor are they pathogenic for vertebrates. The baculovirus expression vector system has been used to express genes derived from viruses, fungi, bacteria, plants, and animals. In this system, foreign genes placed under the control of the strong polyhedrin promoter of the AcMNPV are usually expressed at high levels in cultured Sf9 cells. Baculovirus constitute one of the largest known groups of viruses, and they are capable of infecting over 500 species of insects, and these viruses have shown the ability to make ideal vectors.

The most widely used lepidopteran cells for BEVS are the Sf9 and Sf21 cell lines isolated from ovarian tissue of the fall army worm, *Spodoptera frugiperda,* and the High Five (HF) cell line. Sf9 is a substrain (clone) of Sf21 cells, isolated from these cells by researchers at Texas A&M University. Both the clone and parent strains of the cells have been extensively used in research on viruses, especially baculoviruses in their use for producing recombinant proteins. Sf9 cells were selected for their faster growth rate and higher cell densities than the Sf21 cells. Either Sf9 or Sf21 are preferred for virus expansion. The replication cycle is biphasic, with gene expression occurring as a cascade of sequentially and temporally regulated events. During these events two different forms of the virus are synthesized: extracellular (budded) virus particles (ECV) for cell to cell infection within the insect and viruses contained in occlusion bodies (OV) for spread to a new host insect.

The various advantages of Baculovirus Expression System that make it an ideal tool for the synthesis of VLPs, which generally require simultaneous expression of multiple viral structural proteins for self-assembly are as the large cloning capacity, production of relatively large quantities of post translational modified eukaryotic proteins, quick turnaround time for the expression of recombinant proteins, recombinant proteins produced show biological activity, antigenicity, and immunogenicity similar to authentic natural proteins, baculovirus does not infect vertebrates and so is safe to animals and environment.

In this context, insect cell based baculovirus system that utilizes eukaryotic type post translational modifications are more suitable (Roy and Noad, 2009). The baculovirus expression system is a valuable expression system that has successfully produced many kinds of virus-like particles from viruses such as enteroviruses (Hu *et al.,* 2003), poliovirus (Urakawa *et al.,* 1989), rabbit hemorrhagic disease virus (Laurent et al., 1994), Norwalk-like viruses (Mortola and Roy, 2004), severe acute respiratory syndrome (SARS) virus (Belliot *et al.,* 2001), swine vesicular disease virus (Ko *et al.,* 2005), type Asia1 and type O viruses of FMD (Cao *et al.,*2010; Mohana Subramanian *et al.,* 2012; Basagoudanavar *et al.,* 2013; Bhat *et al.,* 2013) and blue tongue virus (Stewart *et al.,* 2013).

Baculovirus-derived VLP experimental FMD vaccines have been shown to provide some protection against clinical disease in swine. Similar results using an *E.coli*-derived VLP experimental vaccine were also reported. There are also reports showing improvements by using baculovirus and silkworm larvae to express FMDV P1 and including protein 2A at strategic sites to facilitate processing of VP1–VP2 and VP0 (Fig. 1). The authors have provided electron microscopy evidence of VLP assembled in the larvae lysates. Furthermore, vaccines prepared in this fashion for serotypes Asia and O conferred protection when used to immunize cattle.

2.8. Different strategies to enhance FMD Virus-like Particles (VLPs) synthesis

There has been some success in using different systems for the production of the FMDV empty capsid particles but there have also been a number of challenges. For example, it has not been possible to isolate single recombinant vaccinia viruses which co-express the P1-2A precursor with the 3Cpro (Abrams *et al.*, 1995). Furthermore, in the baculovirus expression system, the 3Cpro has proved to have adverse effects on protein expression (Porta *et al.*, 2013b). This may result from the fact that the FMDV 3Cpro has a number of cellular targets including certain translation initiation factors (eIF4G and eIF4A) (Belsham, 2000), cytoskeleton components (Armer *et al.*, 2008) and histone H3 (Falk *et al.*, 1990). Indeed, constitutive expression of FMDV 3Cpro can only be achieved at low levels in mammalian cells (Martinez-Salas and Domingo, 1995). This has led to attempts to decrease the level of 3Cpro activity that is co-expressed with the P1-2A precursor and it has been shown that equimolar amounts of the 3Cpro are not required to achieve efficient processing of the P1-2A, by co-transfecting plasmid containing structural gene and different concentration of 3C plasmid within both insect and mammalian cells (Polacek *et al.*, 2013; Porta *et al.*, 2013a; Porta et al., 2013b).

One strategy to reduce the level of 3Cpro activity is to use mutant forms of this protease with reduced enzymatic activity (Sweeney *et al.*, 2007). An alternative system relies on reducing the amount of 3Cpro expression relative to the P1-2A. This can be achieved by differential levels of transcription, e.g. using different promoters to drive the expression of two different cDNA cassettes as used with enterovirus 71 (Chung *et al.*, 2010) and FMD Asia 1 (Cao et al., 2009). Alternatively, a single cDNA cassette containing the coding sequences for FMDV P1-2A and 3Cpro has been used in insect cells with the two coding sequences being separated by a translational frame shift signal sequence (Porta *et al.,* 2013a; Porta *et al.,* 2013b). In addition, two separate open reading frames (ORFs) can be expressed from a single bi-cistronic mRNA with an inefficient (mutant) internal ribosome entry site (IRES) located between them. The use of a mutant IRES (designated mIRES) can result in relatively low level translation of the downstream ORF (here 3Cpro) (Gulberg *et al.,* 2013). Moreover, presence of an IRES in the dicistronic mRNA not only drives expression of the downstream reporter gene but also positively influences first transgene expression (Jünemann *et al.,* 2007). Borman *et al.,* (1997) compared the capacities of different picornaviral IRESes to mediate internal translation initiation in a variety of cultured cells. EMCV and FMDV, which were 60–80% efficient in directing internal translation initiation in Hela cells. EMCV element, which was considerably less efficient than the FMDV or HCV IRESes in BHK21, suggesting that IRES will have different translational efficiency in different cell types.

Keeping in view of the above literature the present study has envisaged with objective of enhancing the structural capsid proteins of FMD virus through moderated 3C protease to form empty capsid which could have antigenic and immunogenic properties for developing newer diagnostics tools and vaccines.

Chapter 3

Materials and Methods

3.1. MATERIALS

3.1.1. Equipments

1. Refrigerated centrifuge
2. Microcentrifuge –minispin (Eppendorf, Germany).
3. Circulating water bath (F12 Julabo, Germany)
4. Deep freezer -80°C (Sanyo)
5. Horizontal and vertical laminar air flow cabinets (Klenzaids, India).
6. Sorvall RT 7 centrifuge.
7. Sorvall Wx ultra 80 centrifuge.
8. Thermal cycler (Veriti, Applied Biosystem).
9. Horizontal electrophoresis unit (SCIE-PLAS, USA)
10. Orbital shaker (MaxQ shaker, Thermo Scientific).
11. Plastic ware and glass ware
12. Incubator (37°C) and CO_2 incubator.
13. Inverted binocular microscope (Nikon-Model TMS, Japan)
14. Weighing balance-BP-121S (Sartorius, Germany)
15. Micropipette of various volumes.
16. ELISA reader Infinite F50 (Tecan).
17. Transblot apparatus (Amersham Biosciences, USA)
18. Vortex mixer.
19. Vertical electrophoresis unit (Biorad, USA).

3.1.2. Cell lines

Spodoptera frugiperda (Sf-21) and *Trichoplusia ni* (Tn5) insect cells were maintained at 27°C in SF-900 II SFM (Invitrogen) and BHK-21 cells maintained in Glasgow's Modified Eagles Medium (GMEM) were used in the study. The human embryonic kidney (HEK-293) cell line (# 240085, Agilent Technologies, USA) was maintained in Eagle's minimum essential medium (# 12-611Q, Lonza, Belzium) containing 2% fetal bovine serum (FBS), 25mM HEPES, nonessential amino acids (Lonza, Belzium), penicillin (100 IU/ml), streptomycin (100 µg/ml) were used in adenoviral expression study.

3.1.3. Viruses

Indian FMD vaccine virus serotype O, (Ind-R2/75 strain), serotype A, (Ind-40/00 strain) and serotype Asia I, (Ind-63/72 strain) adapted in BHK-21 cells, was used for viral RNA extraction (Qiagen RNA Easy Mini Kit, Germany) and to synthesize cDNA (Thermoscript RT kit, Invitrogen, USA) for use in polymerase chain reaction (PCR) to amplify genomic sequences.

3.2. METHODS

3.2.1 Primer design and gene synthesis

Based on the genomic sequence of Indian FMD vaccine virus, AcGFP and EMCV IRES, primer pairs (Table 1) were designed with restriction enzyme sites to facilitate cloning in proper reading frame with respect to their transfer vectors (pFastBAC-1, pFastDUAL, pShuttle CMV vector) using the SnapGene software (version 2.5).

PCR mediated mutagenesis was performed for the 3C cleavage protease gene (3Cpro^{wt}) at two positions (G38S and F48S) using the primer pair G38F48S-F: 5'-AGCACTGCCTACCTCGTGCCTCGTCATCTTTCCGCAGAGAAG-3' and G38S48S-R: 5'-GGAAAGATGACGAGGCACGAGGTAGGCAGTGCTGAACACTCC-3' and the amplicon was designated as mutant 3C protease (3Cpro^{mut}). Synthesis of 3Cpro^{mut} was carried out in two steps of the mutagenic PCR. The Step I involves synthesis of two regions 3Cpro^{mut1} and 3Cpro^{mut2} spanning the complete 3Cpro^{mut}. cDNA template (FMDV Type O) and primer pair (SpeI-3BC-F and G38S-F48S-R) were used in a PCR reaction to amplify 3Cpro^{mut1} fragment; while the cDNA template (FMDV Type O) and primer pair (G38F48S-F and SphI-3C-R) were used in a PCR reaction to amplify 3Cpro^{mut2} fragment. In step II, both the fragments as template and primer pair (SpeI-3BC-F and SphI-3C-R) were used in a PCR reaction to amplify 3Cpro^{mut} (overlap PCR).

Table 1

a. OLIGONUCLEOTIDE PRIMERS USED FOR AMPLIFICATION OF TARGET GENE

Target gene	Primer	Primer Sequence (5'→3' direction)	Restriction enzyme Site	Fragment Length
FMDV P1-2A IND R2/75	BamHI-VP4-F	TG*GGATCC*ATGGGAGCYGGGCAATCCAG	*BamHI*	2318 bp
	SalI-VP4-F	TG*GTCGAC*ATGGGAGCCGGGCAATCCAG	*SalI*	
	SpeI-2B29R-STOP-R	TG*ACTAGT*CTACACCAGYTTGGAGAAGT	*SpeI*	
	SpeI-2B29R-R	TG*ACTAGT*GCTAAGAACCTGATTGTCACTGAGAGTG GTGCCCCRC	*SpeI*	
FMDV_O_IRES IND-R2/75	SpeI-OA-IRES-F	TG*ACTAGT*AGCAGGTTTCCCCAAC	*SpeI*	463 bp
	SpeI-O-IRES-R	TG*ACTAGT*TGAAAGTAGTAGTRRTTAATG	*SpeI*	
FMDV_A_IRES IND-40/00	SpeI-OA-IRES-F	TG*ACTAGT*AGCAGGTTTCCCCAAC	*SpeI*	460 bp
	SpeI-A-IRES-R	TG*ACTAGT*CAGTTTAGTAATGGTAAAAGA	*SpeI*	
FMDV_As_IRES IND-63/72	SpeI-As-IRES-F	TG*ACTAGT*ATGCAGGTAGCCCCAACTGAC	*SpeI*	462 bp
	SpeI-As-IRES-R	TG*ACTAGT*GTAAACAGTAGTGGTCAAAGG	*SpeI*	
EMCV IRES	SpeI-EMCV-IRES-F	TG*ACTAGT*AATTCCGCCCCTCTC	*SpeI*	581 bp
	SpeI-EMCV-IRES-R	TG*ACTAGT*TTATCATCGTGTTTTTC	*SpeI*	
FMD 3Cpro IND R2/75	SpeI-3B3C-F	TG*ACTAGT*ATGGCTAAGAACCTGATTGTCACTGAGAG TGGTGCCCCGC	*SpeI*	649 bp
	SphI-3C-R	TG*GCATGC*CTACTCRTGGTGTGGTTCGG	*SphI*	
	EcoRV-3C-R	CG*GATATC*CTACTCATGGTGTGGTAC	*EcoRV*	
AcGFP	SpeI-AcGFP-F	CA*ACTAGT*ATGGTGAGCAAGGGCGCCGAG	*SpeI*	736 bp
	SphI-AcGFP-R	CC*GCATGC*TCACTTGTACAGCTCATCCAT	*SphI*	

b. VECTOR BINDING PRIMERS FOR SEQUENCING

Vector	Primer	Primer Sequence (5'→3' direction)
Baculoviral	M13-F	CCCAGTCACGACGTTGTA AAACG
Bacmid	M13-R	AGCGGATAACAATTTCACACAGG
pFastBac-1	BAC-F	GGAGATAATTAAAATGATAACCATCTCGC
pFastBac-Dual	BAC-R	GGGAGGTGTGGGAGGTTTTTTAAAGCAAG
pFastBac-Dual	P10-F	GTATATTAATTAAAATACTATACTG
	P10-R	TCCTTCCGTGTTTCAGTTAGCCTCC

*Restriction enzyme recognition sequence underlined in primer sequence.

3.2.2. Construction of recombinant baculovirus transfer plasmids

The following recombinant vectors containing capsid region and 3C protease of FMDV serotype O were constructed to generate recombinant baculovirus transfer plasmids. Two approaches were employed: (1) use of IRES intervening sequences between P1-2A polyprotein and 3C protease under single promoter and (2) use of dual promoter system to transcribe P1-2A and 3C. They are explained here under.

3.2.2.1. Recombinant pFastBac1 construct for unidirectional-bicistronic expression using viral IRES sequences.

Virus capsid coding region of P1-2A (2.3 kb) and non-structural protein 3C genomic sequence (639 bp) were amplified from cDNA of FMDV serotype O using specific primer pairs (Table 1). IRES elements were amplified from cDNA of all three FMDV serotypes (O, A and Asia 1). The following constructs were prepared and cloned into pFastBac 1 transfer vector to generate unidirectional bicistronic expression using FMD-IRES sequence (Fig. 2). Construct 1: To check the translational effectiveness of FMDV IRES mediated protein expression in the construct, AcGFP gene (2nd transgene) was inserted downstream of FMDV IRES sequence to visualise the green fluorescence. IRES elements of three serotypes of FMDV (O, A and Asia I) were inserted upstream of second transgene to generate the constructs (Fig. 2).

Construct 2: P1-2A and 3Cpro^{wt} intervened with FMDV IRES. IRES elements of three serotypes of FMDV (O, A and Asia I) were individually constructed to compare efficiency.

Construct 3: P1-2A and 3Cpro^{mut} intervened with FMDV IRES.

EMCV IRES sequence amplified from pBICEP 3 plasmid were used here to generate the following constructs in pFastBac 1 transfer vectors.

Construct 4: To check the translational efficiency of EMCV IRES mediated protein expression in the construct, AcGFP gene (2nd transgene) was inserted downstream of FMDV IRES sequence to visualise the green fluorescence.

Construct 5: P1-2A and 3Cpro^{wt} intervened with EMCV IRES (Fig. 2).

3.2.2.1.1 Characterization of recombinant transfer vector construct

The genomic sequence cloned within the transfer vector (recombinant) was confirmed for the presence of gene of interest by using appropriate restriction enzyme site digestion (Fig. 2) and also by sequence analysis bi-directionally using vector binding primer pair (Table 1) to verify the direction and correctness of their reading frame prior expression studies. The sequencing was done by using the automated DNA Sequencer at the Sequencing Facility, M/s Eurofins, Bangalore. The data obtained was analyzed for the presence of gene of interest (GOI) using BLAST, Mega 6 sequence alignment tool and also analyzed for the presence of RE site using SnapGene 2.5 software for further use in cloning. The FMDV IRES sequences which were used in construction of recombinant plasmid were sequence analysed and submitted in GenBank.

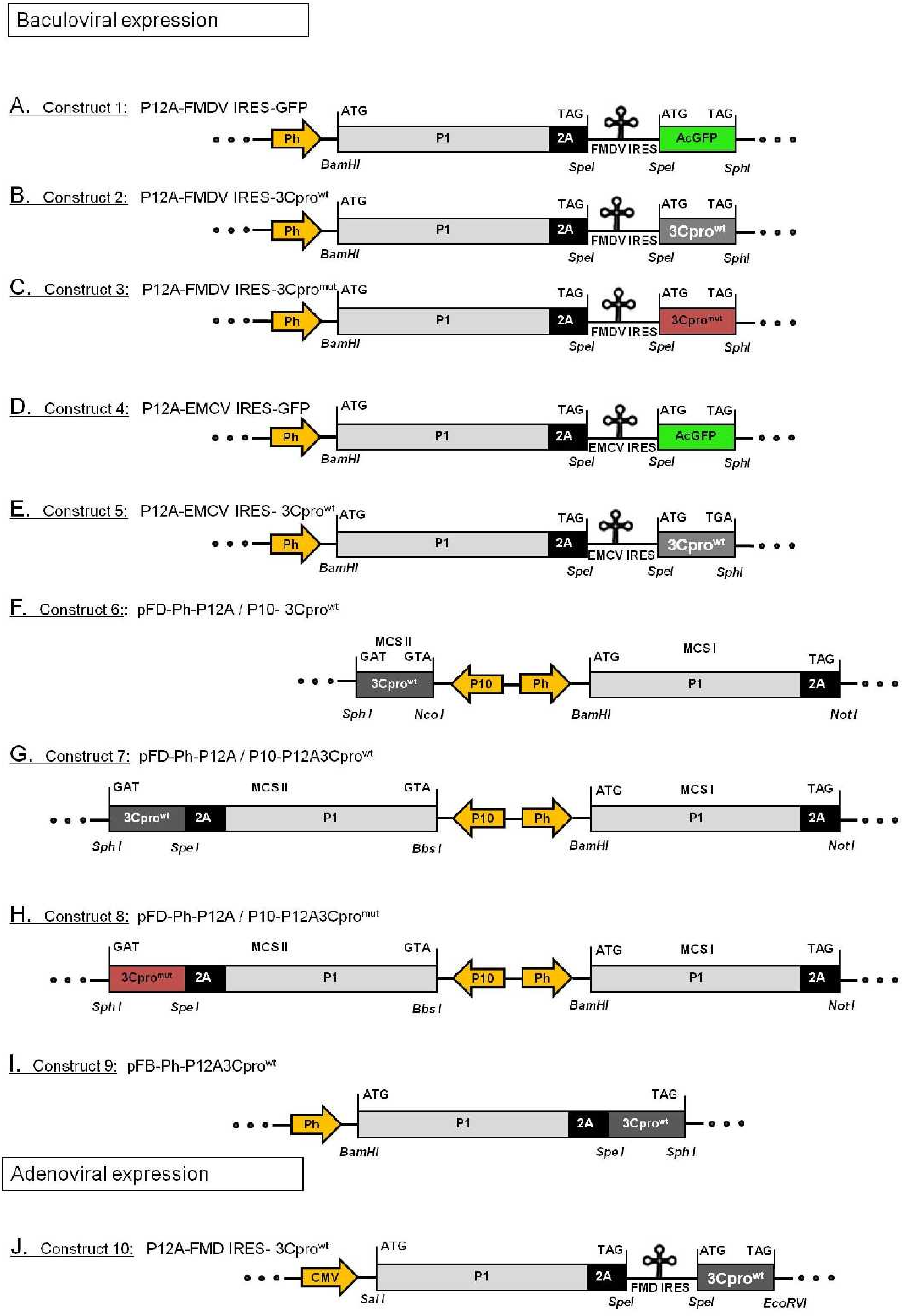


Fig. 2. Schematic representation of the cDNA cassettes constructed in this study for the expression of two different genes (P12A and 3Cpro^{wt}/3Cpro^{mut}/AcGFP) simultaneously. Ph, Polyhedrin promoter; P10, P10 promoter; P1-2A, Capsid precursor protein (FMDV serotype O); 3C, 3Cpro; ATG, start codon; TAG, stop codon.

3.2.2.3. Recombinant pFastDual construct for bidirectional-bicistronic expression

The following recombinant pFastDual transfer vectors containing capsid region and 3C protease (3Cpro^{wt})/ mutant 3C protease (3Cpro^{mut}) of FMDV serotype O were constructed to generate bidirectional-bicistronic expression.

Construct 6**:** P1-2A was cloned under polyhedrin promoter (MCS-I) while 3Cpro^{wt} was cloned under P10 promoter (MCS-II) into pFastDual vector (Invitrogen) (Fig. 2).

Construct 7**:** P1-2A region cloned under polyhedrin promoter (MCS-I) while, P1-2A fused with 3Cpro^{wt} as a single transcriptional unit (without stop codon) was cloned in MCS-II under the p10 promoter. For this, amplicons using VP4-F and 2B29R-SPEI-R and 3B3C-F and 3C-R were fused in *SpeI* enzyme site without any transcriptional stop between these two sequences (Fig. 2).

Construct 8**:** P1-2A region cloned under polyhedrin promoter (MCS-I) while, P1-2A fused with 3Cpro^{mut} as a single transcriptional unit (without stop codon) was cloned in MCS-II under the p10 promoter of pFastDual vector (Fig. 2).

Another construct to express P1-2A-3C as single transcriptional unit was made.

Construct 9**:** P1-2A-3C sequence as single transcriptional unit, but cloned under polyhedrin promoter into pFastBac-1 vector (Invitrogen) to generate a unidirectional-monocistronic expression (Fig. 2).

Expression level of the above first eight constructs was compared with the construct 9 as this construct has been earlier shown to form VLPs in this lab and tested as a diagnostic reagent.

All the recombinant baculoviral transfer plasmids generated were confirmed for the presence of gene of interest (GOI) as described earlier in section 3.2.2.1.1.

3.2.3. Generation of recombinant bacmid DNA clones.

Recombinant transfer vectors were transformed into DH10Bac *E.coli* cells (Invitrogen) to produce the recombinant bacmid by site-specific transposition. DH10Bac *E.Coli* cells were made competent for transformation by the calcium chloride method as per the standard protocol (Sambrook and Russell, 2001), keeping the final concentration of sterile glycerol to 15-30% and stored at -80˚C for the future use.

3.2.3.1. Transformation of recombinant baculovirus transfer plasmid into DH10Bac competent cells

Transformation was done as per the modified standard protocol described in Sambrook and Russell (2001). Briefly the frozen aliquots *E. coli* (DH10Bac) competent cells were thawed on ice and mixed with purified recombinant plasmid (0.5 µl) and incubated on ice for 30 minutes with intermittent mixing gently. The cells were given heat shock at 42˚C for 60 seconds followed by snap cooling on ice for 5 minutes. Immediately thereafter 900 µl of SOC broth (Appendix) was added to the cells and the suspension was incubated at 37˚C for 5 hours in shaking incubator (220 rpm). The cells were placed on LB agar plates (containing 100mM IPTG, Bluo-gal 40 µg/ml, kanamycin 50 µg/ml, gentamicin 7 µg/ml, and tetracycline 10 µg/ml) and incubated at 37˚C for 24-48 hours. The positive clones identified as white colonies were screened by colony PCR using gene specific primer pair. The clones which were positive by colony PCR are inoculated into 2 ml LB medium supplemented with kanamycin (50 µg/ml), gentamicin (7 µg/ml), and tetracycline (10 µg/ml) and incubated at 37°C in shaker incubator (250 rpm).

3.2.3.2. Bacmid isolation and analysis of recombinant bacmid DNA clone

The recombinant baculoviral bacmid from the bacterial culture was isolated using Qiagen plasmid DNA isolation kit and confirmed for the presence of gene of interest in the recombinant bacmid by PCR using the M13 Forward and M13 Reverse primer pair that hybridize to sites flanking the mini-attTn7 site within the lacZα-complementation region. If transposition has occurred; PCR product of the following size on the agarose gel could be observed.

Sample	Size of PCR product
Bacmid alone	~300 bp
Bacmid transposed with pFastBac-1	~2300 bp + size of the insert
Bacmid transposed with pFastBac-Dual	~2560 bp + size of the insert

3.2.4. Generation of recombinant baculovirus and expression of FMDV capsid proteins

The recombinant bacmid DNA constructs isolated were transfected into Sf-21 insect cells to produce recombinant baculoviruses. Viruses were propagated 2-3 rounds to amplify the titers of seed virus in Sf-21 insect cells and were analyzed for expression of the recombinant proteins upon infection of Tn5 cells.

3.2.4.1. Transfection of insect cells and preparing the P1 viral stock

Transfection was carried in a 6-well plate cultured with Sf-21 insect cells (1.5×10^6 cells per well) in SF-900 II SFM (Invitrogen) containing 10% FBS. For transfection, antibiotic free SF-900 II SFM was used. While cells were allowed to adhere to the surface, following transfection mixtures were prepared.

Mix A: SF-900 II SFM–100ul mixed with Cellfectin-II (Invitrogen)-8ul and incubated for 5 minutes. In the mean time, prepare the following mix

Mix B: SF-900 II SFM -100ul mixed with 5ul of bacmid DNA isolated as above. Both the mixtures are mixed together and incubated for 20-25 minutes.

In the mean time, cells were gently washed twice with SF-900 II SFM to get rid of dead cells and FBS. Transfection mixture containing the celfectin-II and bacmid DNA is added onto cells (Care is taken to avoid cell drying during the washing or replacing the medium with above transfection mix) and incubated at 27°C for 5 hours. After incubation; transfection mixture was removed and replaced with 2 mL of complete growth medium (SF-900 II SFM supplemented with 10% FBS) and incubated further at 27°C for 72 hours or until signs of viral infection. Then the medium containing virus from each well (~2 mL) were collected and transferred to sterile 2ml eppendorf tubes. The tubes were centrifuged at 500 × g for 5 minutes to remove cells and debris. This is the P1 viral stock stored at 4ºC. Recombinant baculoviruses were propagated 2-3 rounds to amplify the viral titers in Sf-21 insect cells and were analyzed for expression of the recombinant proteins upon infection of Tn5 insect cells.

3.2.4.2. Expression of FMDV capsid proteins in Tn-5 cells

Tn-5 insect cells, infected with recombinant baculoviruses at multiplicity of infection 5 (7×10^7 pfu/ml i.e. high-titer rAcNPV stock) were incubated at 27°C for 3 days. Infected cells were then recovered by pelleting (192 x g for 10min) and lysed in lysis buffer (50mM Tris, 100mM NaCl, containing 0.1% Triton X 100 and protease inhibitors). The clear lysate was obtained by sonicating and supernatant collected.

3.2.5. Characterization of the recombinant baculoviruses expressing FMDV capsid protein

Expressed recombinant proteins from baculoviral expression system were analysed by SDS-PAGE, Western blot and Sandwich ELISA.

3.2.5.1. SDS-PAGE analysis

Expressed recombinant proteins boiled with SDS buffer (1X) for 10 min and subjected to SDS-PAGE analysis as per the modified protocol described by Laemmli (1970) using 13% and 5% of separating and stacking gels, respectively along with prestained protein ladder (Thermo scientific cat no. 26616, Lithuania) followed by Coomassie Brilliant Blue-R250 (CBB) staining.

3.2.5.2. Western blot analysis

After SDS-PAGE, the expression of recombinant protein was confirmed by Western blot analysis as per the standard protocol (Sambrook and Russell, 2001). Proteins from the gel were blotted onto a PVDF membrane using Transblot apparatus. The PVDF membrane was treated with blocking buffer (Appendix) at 37˚C for 1 hour. After blocking, the membrane was washed three times with PBS-T solution (Appendix) at 4 minutes interval each and then transferred to a container containing of rabbit antiserum (dilution 1:500) raised against FMDV type O (Ind-R2/75 strain) 146S. On incubation at 4˚C for overnight the membrane was washed thrice with PBS-T (Appendix) as earlier and then incubated at 1:1000 concentration of polyclonal goat anti-rabbit immunoglobulin HRP conjugate (DAKO, Denmark) in blocking buffer at 37˚C for 1 hour in shaker incubator (60 rpm). The membrane was washed thrice with PBS-T after incubation to eliminate unbound conjugate. After washing, 1X substrate buffer (Appendix) containing DAB and H_2O_2 was applied to the membrane and kept in dark for 20 seconds for colour development. The colour reaction was stopped by distilled water and the membrane was visualized.

3.2.5.3. Sandwich ELISA

- ELISA plates (Maxisorp, Nunc) were coated with serotype specific rabbit anti-146S serum in carbonate-bicarbonate buffer pH 9.6 at 4°C, overnight. Coating was done by adding 50 ul of serotype specific rabbit anti-146S serum in 2 horizontal rows for each serotype (A1-A12, B1-B12 for O and like-wise for A and Asia1). The plates were washed thrice with phosphate buffered saline (PBS) containing 0.05% Tween-20.
- A pre-fixed dilution of inactivated FMDV antigen, diluted in Dulbecco's PBS, was added in A1, B1 for O, C1, D1 for A and E1, F1 for Asia1. Each test sample is tested against all 3 serotypes by adding 50 ul of sample column wise. In the last column, blocking buffer was added as negative control wells and incubated at 37°C for an hour.
- Plates were washed thrice and incubated with serotype-specific guinea pig anti-140S serum in blocking buffer in respective rows as used for coating and incubated at 37°C for an hour.
- After washing, anti guinea pig IgG horseradish peroxidase (HRP) conjugate (Dako, Denmark) was added at a dilution of 1:3000 and incubated at 37°C for an hour.
- After final washing, freshly prepared orthophenylene diamine/hydrogen peroxide chromogen substrate was added and allowed for 15 min for colour to develop. Then the reaction was stopped using 1M H_2SO_4. The absorbance in the plates was read at 492 nm using ELISA plate reader (Tecan Infinite50).

The test is valid when all positive control antigen wells show OD more than 1.0 and negative control wells OD less than 0.1. The sample is considered to be positive for a specific serotype when they show more than 3 times the OD against other serotypes and other serotypes OD is less than 0.1 (OIE).

3.2.6. Functional characterization of FMDV IRES in mammalian cells using adenovirus vector system.

The pShuttle-CMV vector containing capsid region, IRES and 3C protease of FMDV serotype O was constructed to generate recombinant adenovirus shuttle plasmid.

3.2.6.1. Recombinant pShuttle-CMV plasmids for unidirectional-bicistronic expression of P1-2A and 3C using FMDV IRES.

The polyprotein coding region of P1-2A (2.3 kb) and 3C viral protease (639 bp) were cloned into pShuttle-CMV plasmid (# 240007, Agilent Technologies). Construct 10: The recombinant pCMV shuttle vectors containing capsid region, IRES and 3Cpro^{wt} of FMDV serotype O were constructed to generate unidirectional- bicistronic expression (Fig. 2).

3.2.6.2. Linearization and transformation of recombinant pShuttle-CMV plasmid into BJ5183-AD-1 competent cells.

The recombinant pShuttle-CMV plasmid construct (pCMV-P12A-IRES-3Cpro^{wt}) was linearized using *Pme I* restriction enzyme (Fig. 3) and transformed into the chemically competent BJ5183-AD-1 cells as per the standard protocol as described in Sambrook and Russell (2001) to generate recombinant adenovirus plasmids (pAd-P12A-AIRES-3C^{wt}) by homologous recombination (Fig. 4). Electroporation competent BJ5183-AD-1 cells (#200157, Agilent Technologies) were used to prepare chemically competent cells as per the protocol described by Sambrook and Russel (2001). The cells were plated on LB agar plates containing kanamycin (50 μg/ml) and incubated at 37˚C for 16-18 hours. The resultant colonies were tested for presence of insert in colony PCR using gene specific primer pair and the positive colonies were grown in LB broth overnight followed by plasmid isolation. The plasmids were further amplified in the Top10 cells by growing in Teriffic broth containing Kanamycin and isolated by plasmid isolation Kit (Qiagen) as per the protocol described by the manufacturer. After isolation of recombinant pAdEasy-1 DNA, the presence of interest was confirmed by restriction enzyme digestion analysis.

Fig. 3. SCHEMATIC REPRESENTATION: CONSTRUCTION OF RECOMBINANT ADENOVIRUS TRANSFER VECTOR

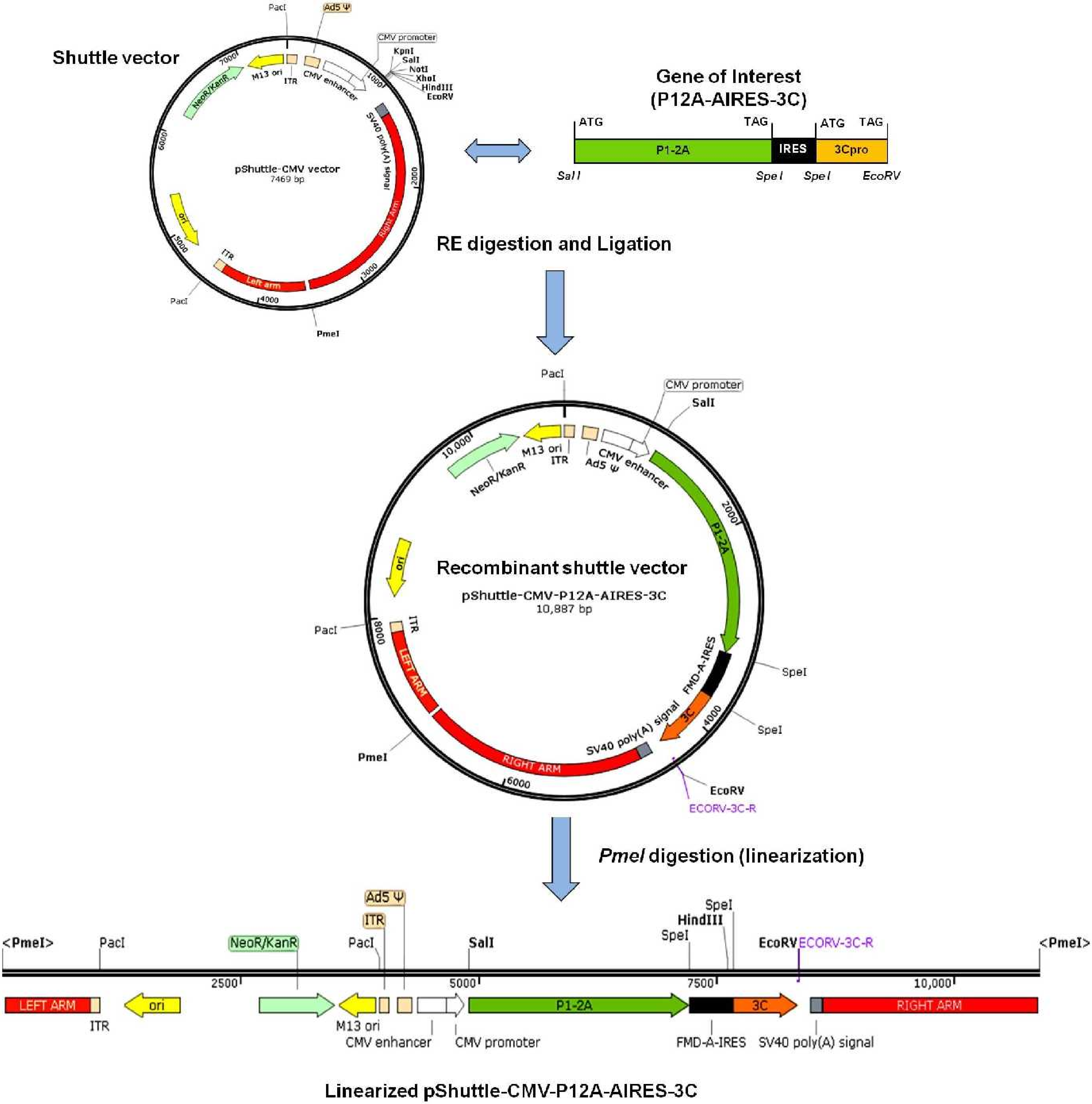
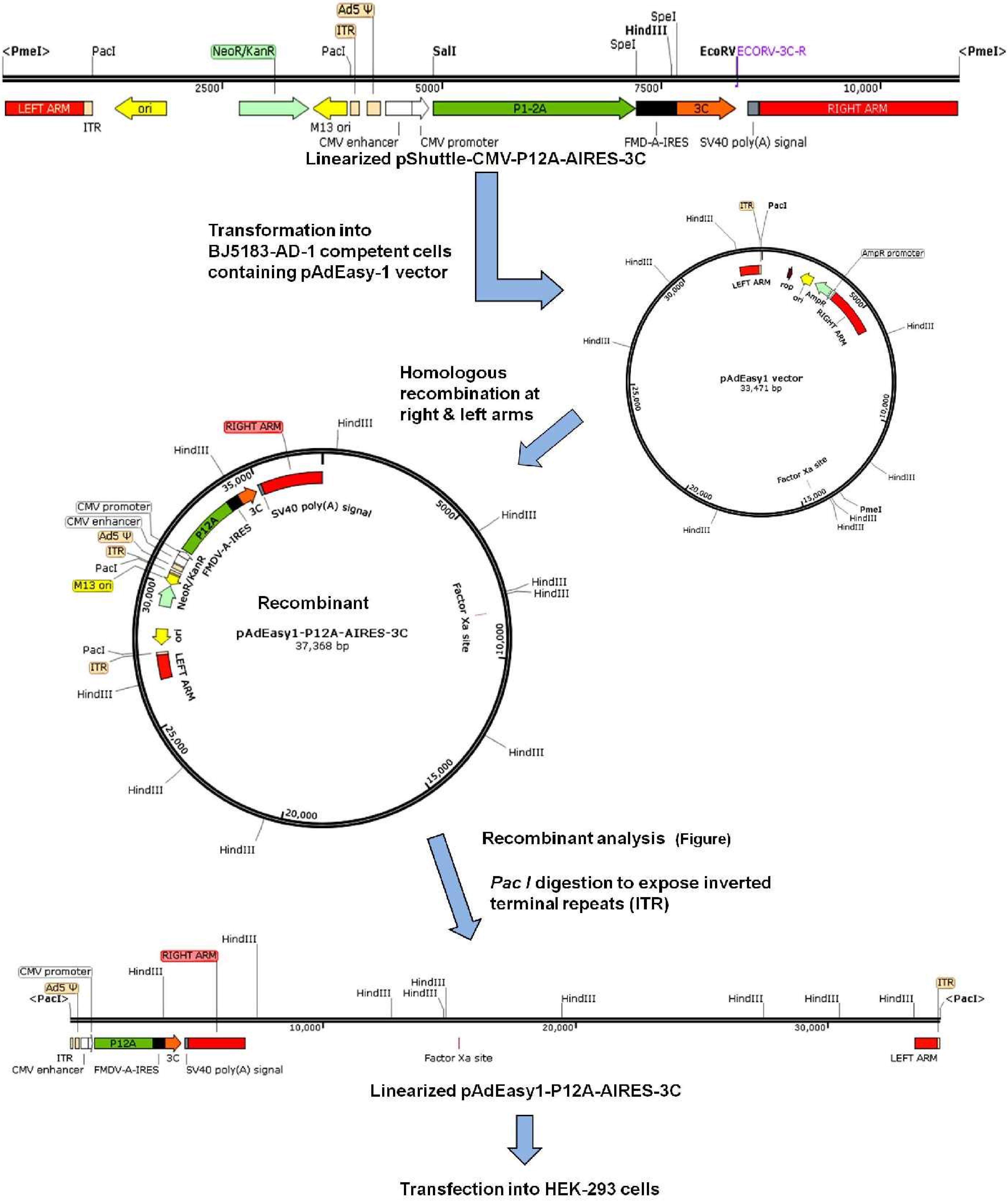


Fig. 4. SCHEMATIC REPRESENTATION: GENERATION OF RECOMBINANT ADENOVIRUS PLASMID



3.2.7. Generation of recombinant adenovirus and expression of FMDV capsid proteins

The recombinant adenovirus plasmids were digested with *PacI* enzyme before transfection into HEK-293 to produce recombinant adenoviruses (Fig. 4). These recombinant adenoviruses were propagated 2-3 rounds to amplify the viral titers in HEK-293 cells and were then analyzed for their expression.

3.2.7.1. Generation of recombinant Adenovirus

The adenovirus plasmids were digested with *PacI* enzyme before transfection in order to expose the inverted terminal repeats (ITR). The HEK-293 cells were cultured in 6 well tissue culture plate (Nunc, Denmark) for 24 hrs to achieve approximately 50% confluency. Transfection of HEK-293 cells with the above adenovirus plasmids (4 µg of the plasmid DNA per well) was performed by using Virapack Transfection Kit as per manufacturer instructions (#200488, Agilent Techologies). The transfected cells were observed daily upto 10 days for the appearance of virus induced plaques. Plaques which appeared early were individually picked up carefully and pelleted by centrifuging at 200g for 10 min. The cells were re-suspended in Dulbecco’s phosphate buffer saline (DPBS) containing Ca++ and Mg++ and freeze-thawed thrice to release the virus. Then the virus-cell suspension was centrifuged at 5000g for 10 min and supernatant containing the virus was collected and stored at -80°C. The plaque viruses were titrated and the viruses having the higher titre were selected to prepare seed viruses. The seed viruses were used for further amplification of the virus. The viruses were serially passaged by infecting the HEK-293 cells at a multiplicity of infection (MOI) of 0.01.

3.2.7.2. Expression of FMDV capsid proteins in HEK293 cells and its confirmation

HEK-293 cells, were infected with recombinant adenoviruses at multiplicity of infection 5, and incubated at 37°C for 3 days. Infected cells were then recovered by pelleting (192 x g for 10min) and lysed in lysis buffer (50mM Tris, 100mM NaCl, containing 0.1% Triton X 100 and protease inhibitors). The clear lysate supernatant collected and used for SDS-PAGE and western blot.

3.2.8. Characterization of the recombinant adenovirus expressing FMDV capsid protein

Expressed recombinant proteins from adenoviral expression system were analysed by SDS-PAGE, Western blot and Sandwich ELISA as described in section 3.2.5.

3.2.9. Purification of expressed VLPs of FMDV type O IND/R2/75

Infected Tn5 cells were collected by centrifugation on 3 days post infection and the pellet lysed by re-suspending in lysis buffer (50mM Tris, 100mM NaCl, containing 0.1% Triton X 100 and containing 1mM each of PMSF) and sonicated on ice (amplitude of 27% for 1 minute with pulse of 10 seconds with pause of 20 seconds). Unbroken cells and nuclei were removed by centrifugation (4500 rpm, 15min) and the clarified supernatant was layered onto a 30% sucrose (w/v in 50mM Tris, 100mM NaCl) cushion. After ultracentrifugation at 100,000g for 100 min (Sorvall WX Ultra series, Thermoscientific), the supernatant was discarded and the pellet suspended in Tris-NaCl and stored at 4^{0}C overnight. Re-suspended pellet was then applied to a preformed discontinuous 30–60% sucrose gradient with the increment of 10% of four gradient (w/v in Tris-NaCl) (2 ml of each 30%, 40%, 50% and 60% sucrose gradient and remaining with sample) in 17ml polyallomer tubes. After ultracentrifugation at 100,000g for 16hr, 4 fractions of 2 ml each were collected from bottom to top using siphon-tube and tested in sandwich ELISA, spectrophotometry and western blotting. Following spectrophotometry, the whole protein concentration (mg/ml) of the collected fractions were estimated as per the formula [1.55 x A280]–[0.76 x A260] (Layne *et al.*, 1957).

3.2.10. VLP quantitative analysis

The baculoviral expressed VLP samples of three different constructs (Construct 2, 7, 9) (Fig. 2) were quantified by sandwich ELISA (Basagoudanavar et al., 2015). In this experiment, cells were infected with recombinant viruses at the same MOI and subjected similarly in the downstream processing. Each well in the 96-well plate was coated with 50µl of FMDV anti-146S serum raised in rabbit at 1 in 1000 dilution in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight. After 3 washes with PBST buffer (0.05% Tween 20 in PBS), the wells were blocked with PBST containing 1% bovine serum albumin (BSA) for 1 h. After washes, serially diluted samples (expressed VLPs from construct 2, 7, 9) were added (50µl/well). In parallel, known amount of purified FMDV O

146s inactivated antigens were 2-fold serially diluted and added to the wells for standard curve generation. After 2 h incubation and 4 washes, The anti-146S guinea pig tracing antibody in blocking buffer (PBST + 5% skimmed milk powder) was then added to each well. The plate was washed after incubation at 37^0C for 1 hr and anti-guinea pig IgG conjugated to HRPO (Dako, Germany) at 1:3000 dilution in blocking buffer was added and incubated. After washing, freshly prepared orthophenylene diamine/hydrogen peroxide substrate was added and incubated at 37^0C for 15 min for colour to develop. Then the reaction was stopped using 1 M H_2SO_4. The absorbance in the plates was read at 492 nm using ELISA plate reader (Tecan Infinite50).

3.2.11. Transmission Electron Microscopy (TEM)

Purified samples (10µl) were allowed to adhere to the copper grids for 5-10min at room temperature followed by a brief water wash (1min) before staining with 1% uranyl acetate for 1min. Excess stain was removed by blotting and the grids examined on a Transmissible Electon Microscopy (FEI Technai, Netherland) facility available at National Center of Biological Sciences (NCBS), Bangalore.

3.2.12. Preparation of inactivated FMDV O antigens

FMDV vaccine strain of serotype O (R2/75) virus was inactivated, concentrated and purified for the further animal experimental studies.

3.2.12.1. Inactivation of FMDV O virus

Briefly, the BHK-21 monolayer cells grown MP6 virus of FMDV type ‘O’ (vaccine strain) were inactivated using BEI (Binary-ethyleneimine) solution (10 ml/L to achieve 3mM final concentration) as per OIE standards under magnetic stirrer at room temperature for 24 hours. This inactivated virus was neutralized by adding sterile 20% sodium thiosulphate to a final concentration of 2% and proceeded for virus concentration by tangential flow filtration using 30kDa cassette (M/s Sartorius Instrument). BEI inactivation of virus was checked by infectivity assay done on BHK-21 cells and observed for the appearance of visible cytopathic effect (CPE), if any. The absence of visible CPE during three blind passages was considered as a proof of inactivation of the virus.

3.2.12.2. Purification and quantification of inactivated concentrated virus

Purification was done by CsCl density gradient method (Bachrach *et al.,* 1964) using 1.42 g CsCl/ml and 1.38 g CsCl/ml in 36ml polyallomer tube at 27,000 rpm for 4 hours at 4˚C. The gradient was fractionated from the bottom of the tube using long siphon tube. Six fractions were collected and absorbance of the fractions was measured at wavelengths of 259 nm and 239 nm. The fraction with maximum absorbance at 259 and minimum at 239 and giving a ratio of 1.3-1.4 at 259/239 were selected and 146S concentrations were calculated at extinction coefficient (E1% 259) of 76. The fractions containing 146S virus particles were pooled together and dialyzed extensively overnight against Tris-NaCl buffer (Appendix) at 4˚C to remove CsCl salt.

3.2.13. Animal Experimentation: Immunization of Guinea pigs

Guinea pigs of 3-6 months age group weighing about 400-600 g of either sex were used in this study. Guinea pigs were divided into three groups with each group having 10 animals. The animals were immunized by intramuscular route with the different vaccine formulations as indicated in Table 2. The vaccinated animals along with controls were maintained in Animal Experimentation Facility, IVRI, Yelahanka, Bangalore. The necessary approval has been taken from Institute Bio-safety Committee and Institute Animal Ethical Committee (No. BT/17/011/94-P10 dated on 19/06/14).

3.2.13.1. Formulation of vaccine

Each animal in Group I were injected with PBS alone and these served as controls (placebo). In the second group, each animal (Group II) received 5µg of purified VLPs in phosphate buffered saline (pH 7.4), emulsified with equal volume of montanide ISA 206 (ISA-206). Similarly, the animals in the third group (Group III) received 1/10th of the cattle dose of conventional vaccine consisting of 1µg of inactivated FMDV O inactivated antigens in phosphate buffered saline and emulsified with equal volume of ISA-206. The FMD O monovalent antigens or purified VLPs antigen and oil adjuvant (ISA Montanide 206, Seppic) were kept on ice before mixing in ratio 1:1. Required quantity of concentrated antigen is then added to the tube containing the oil and allowed to mix thoroughly to form a milky white oil emulsion (W/O/W). The volume of vaccine administered to each animal was 250 µl. The freshly prepared vaccine is stored at 4°C before dispensing.

Table 2

Immunization groups

GROUPS	NO. OF GUNIEA PIGS	ROUTE	VACCINE FORMULATIONS
I	**10**	**I/M**	Phosphate buffer saline, pH 7.4 + ISA 206 (50%)
II	**10**	**I/M**	Purified VLPs (5µg) + ISA 206 (50%)
III	**10**	**I/M**	FMD O monovalent antigens (2µg) + ISA 206 (50%)

3.2.13.2. Immunization of guinea pigs and preparation of sera

Three groups of 10 guinea pigs in each were used. The vaccines prepared were injected on day 0 and booster dose given on 28 day post vaccination (dpv) by i/m route on the thigh muscle. Animals were bled on 14, 28, 42, and 56 dpv respectively. Following immunization blood samples were collected at different intervals from both primary and booster immunized guinea pigs by intra-cardiac route. Serum was collected in 2ml cryovial tubes after centrifuging at 4500 rpm for 25 minutes at 4˚C and stored at -20˚C. All the serum samples were complement inactivated at 56˚C for 20 minutes.

3.2.14. Evaluation of the immunogenicity

Recombinant proteins were evaluated for their immunogenicity in guinea pigs and the collected sera post vaccination at different intervals were subjected to VNT to determine FMDV neutralizing antibody titres.

3.2.14.1. Virus neutralization test

Sera collected from guinea pigs at 0, 14th, 28th, 42nd and 56th days post immunization were tested for virus neutralization activity by using BHK-21 in microtitre plates as per OIE protocol. The sera was diluted in GMEM maintenance medium in two fold dilution starting from 1:8 to 1:1024 in flat bottoms tissue culture 96 well plates (NUNC) @ 50µl per well. Virus suspension containing 100 $TCID_{50}$ was added to each well and the plates were incubated at 37˚C for 1 hour. 50µl of BHK-21 ($1x10^6$cells/ml) was then added to each well and further incubated in a humidified chamber containing 5%CO_2 at 37˚C. Appropriate serum, virus and cell controls were incubated in the test. After 48 hours of incubation at 37˚C, the wells were observed under microscope for the presence/ absence of virus specific CPE such as rounding and detachment of cells.

3.2.14.2. Indirect ELISA

1. Indirect ELISA was performed on serum samples collected from the experimental guinea pigs at different time intervals.
2. Polystyrene 96 wells microtitre plates (NUNC) were coated with 1:500 dilution of FMDV type 'O' hyper immune sera raised in rabbit (50µl/well) in coating buffer at 37˚C for 1 hour.
3. After wash thrice with PBST solution at 3 minutes interval, the wells were blocked with 5% ABS prepared in PBS-T solution (50µl/well) and incubated at 37˚C for 1 hr.
4. After incubation, plates were again washed as described above
5. Then the plate was added with 1:10 dilution of purified FMDV type 'O' cell culture antigen (50µl/well) in PBS buffer at 37˚C for 1 hour.
6. After wash thrice with PBST solution at 3 minutes interval each, the plates were then blocked with 5% ABS once again prepared in PBS-T solution (50µl/well) and incubated at 37˚C for 1 hr.
7. Each test serum sample was diluted (1:25) in blocking buffer (PBST+ 5% adult bovine serum).
8. 50µl of serum samples (1:25 in PBST solution) were added in duplicate and then kept at 37˚C for 1 hour. FMDV type 'O' hyper immune sera raised in guinea pigs was used as positive control and 0 day sera collected from guinea pigs was used as negative control.
9. Following washing with PBST solution at 2 minutes interval each, 1:3000 dilutions of peroxidase conjugated anti-guinea pig (DAKO) was added. The plates were then incubated at 37˚C for 1 hour.
10. Washing of the plates was done as described earlier and then 50µl/well OPD substrate (3mg in 6 ml citrate buffer pH 5.0 and 2.4µl H_2O_2) was added for color reaction.
11. The reaction was stopped with addition of 1 M H_2SO_4 (50µl/well). Absorbance of the plates was measured at 492 nm by Bio-Rad ELISA reader.

3.2.14.3. Statistical analysis

Statistical significance of differences was evaluated by Student's t test, unless otherwise noted. Differences were considered significant for $p < 0.05$.

Chapter 4

Results

4.1. Primer design and gene synthesis

The polyprotein coding region of P1-2A (2.3 kb) and 3C viral protease (649 bp) were amplified from viral cDNA of FMDV serotype O (Fig. 5) using specific primers as listed Table 1. Viral 3C protease sequence was mutated by site directed mutagenesis using over lap PCR at two positions (G38S and F48S) and was designated as mutant 3C protease ($3Cpro^{mut}$). IRES elements (~462 bp) were amplified from cDNA of FMDV serotypes (O, A and Asia 1). AcGFP (736 bp) sequence was amplified from the pcDNA-AcGFP plasmid vector (Kolangnath *et al.*, 2014), while EMCV IRES (581 bp) sequence was amplified from the pBICEP 3 plasmid vector (Sigma, USA) using primer pairs mentioned in Table 1.

4.2. Construction and confirmation of recombinant baculovirus transfer plasmids

The recombinant baculoviral transfer vectors containing capsid region and 3C protease of FMDV serotype O mentioned in section 3.2.2 were constructed. All the constructs cloned into the transfer vector, either pFastBac dual (pFD) or pFastBac 1 (pFB) were analyzed by restriction enzyme digestion (Fig. 6) and confirmed by nucleotide sequencing (Fig. 7) to verify the gene integrity, direction and correctness of their reading frame. These recombinant transfer vectors were transformed into DH10Bac E.coli cells (Invitrogen) to produce the recombinant bacmid by site-specific transposition based on blue-white colony screening and antibiotic selections.

4.3. IRES sequence analysis

The IRES sequences of FMDV serotypes used in construction of recombinant plasmid were submitted in GenBank (Accession numbers: KP019381 for type O, KP019382 for type A, KP019383 for Asia-1. The Sequence of FMD IRES (3 serotypes) and EMCV IRES were aligned by clustalW and the percent identity of 92.83%, 91.99%, 89.78% alignment score was observed between O: A, O: Asia1 and A: Asia1 IRES, respectively (Fig. 8). Similarly, we could observe only 48-52% alignment score between FMD IRES (3 serotypes) and EMCV IRES. RNA secondary structures prediction analysis (http://rna.tbi.univie.ac.at/) was carried out to determine the structural analogy between

FMDV and EMCV (Fig. 9) and sequence identity was conserved at different motifs of the FMDV and EMCV IRES (Table 3).

4.4. Generation of recombinant baculovirus and Expression of FMDV capsid proteins in insect cells

The representative recombinant baculoviral bacmid DNA constructs isolated were transfected into Sf-21 insect cells using cellfectin to recover the recombinant baculoviruses. The titer of the initial viral stock obtained from transfecting Sf21 cells ranged from 1×10^6 pfu/ml. Recombinant viruses were grown in cell cultures by infecting cells with less than 0.1 MOI to amplify to high titers by serial passaging in Sf-21 cells.

To determine the optimal time of harvesting the recombinant capsid proteins, infected Tn5 cells were collected every 24 hours and subjected to western blot. For this, four T25 flasks each containing 3.5×10^6 cells were infected with MOI of 5 and incubated. Cells were harvested every 24 hours and stored in –80 before analysis of all the samples in western blot and sandwich ELISA. By western blot and sELISA analysis, the maximum expression of capsid protein was observed at 72 hours post-infection (PI) (Fig. 10).

4.5. Characterization of the recombinant baculoviruses clones expressing FMDV capsid protein

Preliminary experiment was carried out with bicistronic expression of construct containing P1-2A and GFP sequence intervened with IRES sequence (Construct 1 and 4) was evaluated. Expression of GFP was observed at few foci of insect cells infected with construct containing P1-2A and GFP downstream of FMDV-IRES sequence (Fig. 11). FMDV IRES was functional in the construct containing P1-2A and GFP or 3C gene. The ~95 kDa capsid polyprotein was successfully cleaved by FMD IRES mediated expressed $3Cpro^{wt}$ as a result, processed structural proteins of VP1/VP3 (~28 kDa) and VP0 (~36kDa) could be observed (Fig. 12, Lane 2, 5; Fig. 13, Fig. 14, Lane 5). This was further confirmed by use of $3Cpro^{mut}$ with reduced 3C proteolytic activity under FMDV IRES showing reduced cleavage of P12A precursors (i.e) increased band intensity of P12A polyprotein and VP3+VP1 intermediate precursor protein (Fig. 12, Lane 3, 6; Fig. 14, Lane 6) or by use of GFP gene with no proteolytic activity showing no cleavage of P12A precursors (i.e) increased band accumulation of P12A polyprotein (Fig. 12, Lane 4, 7).

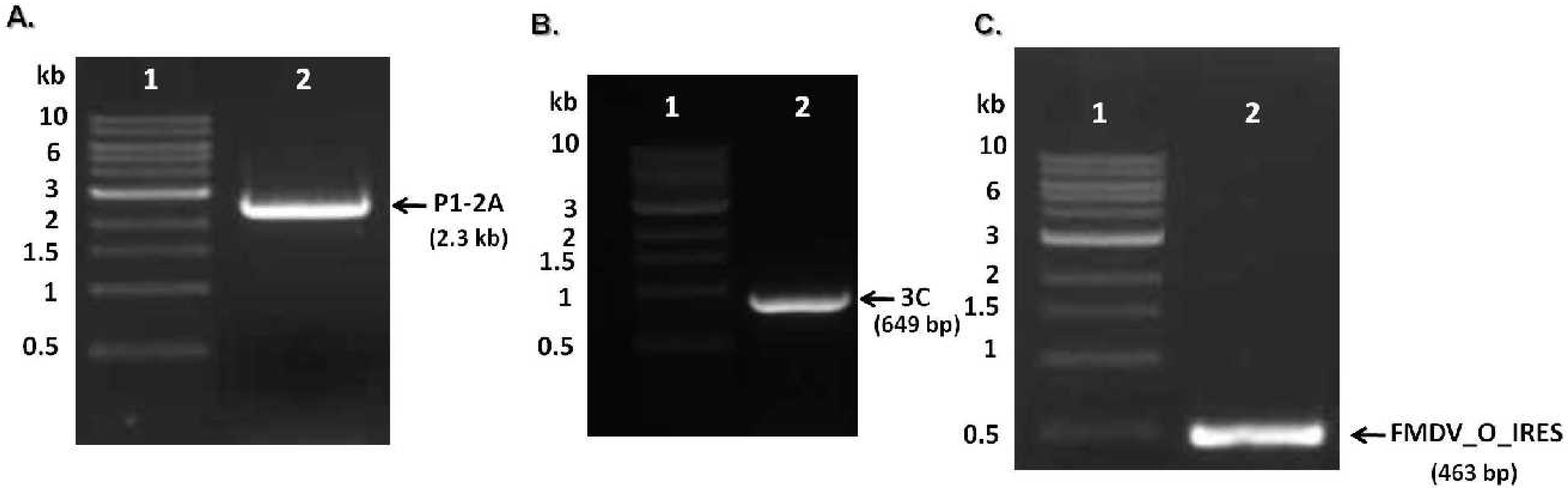


Fig 5. PCR amplification of **A.** FMDV P1-2A, **B**. FMD 3C protease gene & **C.** FMD_O_IRES using specific primers as listed in Table 1.

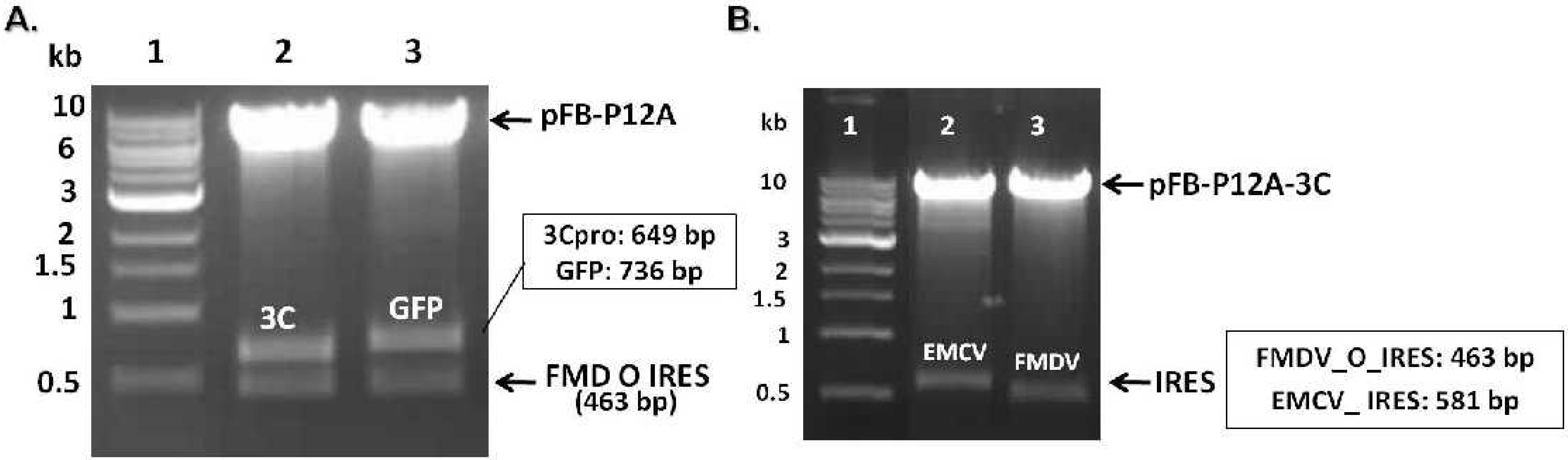


Fig. 6. Restriction enzyme digestion analysis to check the presence gene of interest in the recombinant baculoviral transfer vector. A. RE digesion using SpeI, SphI enzymes. Lane 1- DNA marker, Lane 2- pFB-P12A-FMD_O_IRES-3C, Lane 3- pFB-P12A-FMD_O_IRES-GFP. **B.** RE digestion using SpeI enzymes. Lane 1- DNA marker, Lane 2- pFB-P12A-EMCV IRES-3C, Lane 3- pFB-P12A-FMD_A_IRES-3C.

Fig. 7. SEQUENCES OF THE TARGET GENE USED IN THIS STUDY

FMDV P1-2A CAPSID POLYPROTEIN GENE (SEROTYPE O)

ATGGGAGCTGGGCAATCCAGTCCGGCGACTGGGTCGCAGAATCAGTCAGGCAACACTGGAA
GCATCATCAACAACTACTACATGCAACAGTACCAGAACTCCATGGACACACAACTTGGTGA
CGAGGCTATAAGCGGAGGCTCCAACGAAGGGTCCACGGACACAACTTCCACCCACACAACC
AACACTCAGAACAACGACTGGTTTTCAAAGCTAGCCAGCTCTGCTTTTAGCGGTCTTTTCG
GCGCCCTTCTCGCCGACAAGAAGACTGAGGAGACCACCCTCCTCGAGGACCGCATCCTCAC
CACCCGCAACGGCCACACCACCTCGACAACCCAGTCGAGTGTTGGGCTCACGTACGGGTAT
GCAACAGCTGAGGATTTTGTAAGCGGACCGAACACCTCTGGTCTCGAGACCAGGGTTGTCC
AGGCAGAGCGCTTCTTCAAAACCCACCTGTTTGACTGGGTCACCAGTGACTCATTCGGACG
CTGCCACCTACTGGAACTTCCAACTGACCACAAAGGTGTCTACGGGCAGCCTGACTGACTCG
TATGCTTATATGAGAAACGGTTGGGACGTCGAAGTCACCGCAGTGGGAAATCAGTTCAATG
GAGGATGCCTGTTGGTAGCTATGGTGCCAGAGCTTTGTTCCCTTCAAAAGAGAGAGCTGTA
CCAGCTCACGCTCTTCCCCCACCAGTTGATCAACCCCCGGACGAACATGACGGCACACATC
ACTGTGCCCTTTGTTGGCGTCAACCGCTACGACCAGTACAAGGTACACAAGCCCTGGACCC
TCGTGGTTATGGTCGTAGCTCCTTTGACTGTCAACACTGAAGGTGCCCCACAAATAAAGGT
ATACGCCAACATCGCCCCTACCAACGTGCACGTTGCGGGTGAGTTCCCTTCCAAAGAGGGA
ATGTTTCCTGGGGCTTGTAGCGAGGGTTACGGCGGTTTGGTGACCACGGACCCGAAGACGG
CTGACCCCGGCCTACGGGAAAGTGTTCAATCCCCCTCGGCAACATGTTGCCCGGGGCGGTTGAC
CAACTTCCTTGATGTGGCTGAGGCGTGCCCTACGTTTCTGCACTTCGAGGGTGACGTGCCA
TACGTGACCACGAAGACAGATTCGGACAGGGTGCTTGCTCAATTTGACTTGTCTTTGGCAG
CAAAGCACATGTCGAACACCTTCCTTGCAGGTCTCGCCCAGTACTACACACAGTACAGCGG
TACCATCAACCTGCACTTCATGTTCACAGGCCCCACCGACGCGAAGGCGCGGTACATGATT
GCATATGCCCCCCCTGGCATGGAGCCGCCCAAAACACCTGAGGCGGCTGCCCACTGCATTC
ATGCTGAATGGGACACAGGGTTGAACTCAAAGTTCACATTTTCAATTCCCTACCTCTCGGC
AGCTGACTACGCGTACACCGCGTCTGACACCGCCGAGACCACAAATGTGCAGGGATGGGTC
TGCTTGTTCCAGATAACACACGGGAAAGCTGAAGGTGATGCCCTGGTTGTGTTGGCCAGTG
CCGGCAAGGACTTTGAGTTGCGCCTACCAGTGGACGCCCGTAGAGAGACCACCTCCCCGGG
TGAGTCAGCTGACCCCGTGACCGCCACTGTTGAGAACTACGGCGGTGAGACACAGGTCCAG
AGGCGCCAACACACGGACGTCTCATTCATTTTGGACAGATTTGTAGAAGTGACGCCAAAAG
ACCAAATTAATGTACTGGACCTGATGCAAACCCCTGCTCACACTCTGGTGGGAGCGCTCCT
TCGTACTGCCACTTACTATTTCGCTGACTTAGAAGTGGCAGTGAAACACAAGGGGAACCTC
ACTTGGGTCCCGAATGGGGCGCCTGAAGCGGCGTTGGATAACACCACCAACCCAACGGCAT
ACCACAAGGCACCACTCACCCGGCTTGCATTGCCGTACACGGCACCACACCGTGTGTTGGC
AACTGTTTACAACGGGAACTGCAAGTACGGTGATGGTTCGGTGACCAACACAAGAGGTGAC
CTACAAGTGTTGGCCCAGAAGGCGGCGAGAGCGCTGCCTACCTCCTTCAACTACGGTGCCA
TCAAAGCTACTCGGGTGACTGAACTGCTTTACCGCATGAAGAGGGCTGAGACGTACTGCCC
CAGGCCTCTTTTGGCCATTCACCCGAACGAGGCCAGACACAAACAGAAGATTGTGGCACCT
GTGAAGCAGCTCCTGAACTTTGAACTGCTCAAGTTGGCGGGAGACGTAGAGTCCAACCCTG
GACCCTTCTTCTTCTCCGACGTTAGGTCAAACTTCTCCAAGCTGGTGTAG

FMDV IRES (SEROTYPE O)

AGCAGGTTTCCCCAACTGACACAAAACCGTGCAACTTGAAACTCCGCCTGGTCTTTCCAGGT
CTAGAGCGGTGACATTTTGTACTGTGCTTGACTCCACGCTCGGTCCACTGGCGGGTGCTAG
TAGTAGCACTGTTGCTTCGTAGCGGAGCATGATGGCCGTGGGAACTCCTCCTTGGCAACAA
GGACCCACGGAGGCCGAAAGCCACGTCCTAACGGACCCATCATGTGTGCAACCCCAGCACGG
CAACTTTACTGTGAAAACCACTTCAAGGTGACACTGATACTGGTACTCAAACACTGGTGAC
AGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGAGACACTCGGGATCTGAGAAGGG
GACTGGGGCTTCTTTAAAAGTGCCCAGTTTGAAAAAGCTTCTACGCCTGAATAGGCGACCGG
AGGCCGGCGCCTTTCCATTAATTACTACTACTTTCA

FMDV IRES (SEROTYPE A)

AGCAGGTTTCCCCAACTGACACACACCGTACAATTTGAAACTCCGCCTGGTCTTTCCAGGT
CTAGAGGGGCGACACTTTGTACTGTGATTGACTCCACGCTCGGTCCACTGGCGGGTGCGAG
TAACAGCACTGTTGCTTCGTAGCGGAGCGTGTTGGCCGTGGGATTCCCTCCTTGGTAACAA
GGACCCACGGGGCCGAAAGCCACGTCCTAACGGACCCAACATGTGTGCAACCCCAGCACGG
CAACTTTACTGTGAAAACCACTTTAAGGTGACACTGATACTGGTACTCAACCACTGGTGAC
AGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGCGACACTCGGGATCTGAGAAGGG
GACTGGGGCTTCTTTAAAAGCGCCCAGTTTAAAAAGCTTCTATGCCTGAATAGGCGACCGGA
GGCCGGCGCCTTTCTTTTACCATTACTAAACTG

FMDV IRES (SEROTYPE ASIA-1)

TGCAGGTAGCCCCAACTGACACAAACCGTGCAACTTGGAACCCCGCCTGGGCTTTCCAGGT
CTAGAGGGGTGACGCCCTTGTACTGTGTTTGACTCCACGCTCGGTCCACTAGCGAGTGTTAG
TAGTAGTACTGTTGCTTCGTAGCGGAGCATGACGGCCGTGGGAATCCCTCCTTGGCAACAA
GGACCCACGGGGCCGAAAGCCACGTCCTGAAGGACCCGTCATGTGTGCAACCCCAGCACGG
CAGCTTTATTATGAAACCCACTTTAAGGTGACACTGATACTGGTACTCAAACACTGGTGAC
AGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGCGACACTCGGGATCTGAGAAGGG
GACTGGGGCTTCTATAAAAGTGCCCAGTTTAAAAAGCTTCTATGCCTGGATAGGCGACCGG
AGGCCGGCGCCTTTCCTTTGACCACTACTGTTTAC

EMCV IRES

AATTCCGCCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGG
CCGGTGTGCGTTTGTCTATATGTGATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGG
GCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCA
AAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAG
ACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGC
CTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCC
ACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAA
GGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGC
ACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGG
ACGTGGTTTTCCTTTGAAAAACACGATGATAA

FMDV 3C PROTEASE GENE (3Cpro^{wt}) (SEROTYPE O)

ATGGCTAAGAACCTGATTGTCACTGAGAGTGGTGCCCCGCCGACTGACTTGCAAAAGATGG
TCATGGGCAACACTAAGCCCGTTGAGCTCATCCTCGACGGGAAGACGGTAGCCATCTGCTG
TGCTACCGGAGTGTTT**GGC**ACTGCCTACCTCGTGCCTCGTCATCT**CTT**CGCAGAGAAGTAC
GACAAGATCATGCTGGACGGCAGAGCTATGACAGACAGTGACTACAGAGTGTTTGAGTTTG
AGATTAAAGTAAAAGGACAGGACATGCTCTCAGACGCCGCGCTCATGGTGCTCCACCGTGG
GAATCCGGTGAGAGACATCACGAAGCACTTTCGTGACACAGCAAGAATGAAGAAAGGCACC
CCCGTTGTCGGAGTGATCAACAACGCCGACGTCGGGAGGCTGATTTTCTCTGGTGAGCCCC
TTACCTACAAAGACATTGTAGTGTGCATGGATGGAGACACCATGCCTGGCCTCTTTGCCTA
CAGAGCCGCCACCAAGGCTGGCTACTGTGGAGGAGCCGTTCTCGCTAAGGACGGAGGTGAC
ACATTCATCGTTGGCACTCACTCCGCAGGTGGTAACGGAGTTGGGTACTGCTCGTGCGTAT
CCAGGTCCATGCTTCTGAAAATGAAGGCACACATCGACCCCGAACCACACCACGAGTAG

FMDV 3C MUTANT PROTEASE GENE (3Cpro^{mt}) (SEROTYPE O)

ATGGCTAAGAACCTGATTGTCACTGAGAGTGGTGCCCCGCCGACTGACTTGCAAAAGATGGT
CATGGGCAACACTAAGCCCGTTGAGCTCATCCTCGACGGGAAGACGGTAGCCATCTGCTGTG
CTACCGGAGTGTTC**AGC**ACTGCCTACCTCGTGCCTCGTCATCT**TTC**CGCAGAGAAGTACGAC
AAGATCATGCTGGACGGCAGAGCTATGACAGACAGTGACTACAGAGTGTTTGAGTTTGAGAT
TAAAGTAAAAGGACAGGACATGCTCTCAGACGCCGCGCTCATGGTGCTCCACCGTGGGAATC
CGGTGAGAGACATCACGAAGCACTTTCGTGACACAGCAAGAATGAAGAAAGGCACCCCCGTT
GTCGGAGTGATCAACAACGCCGACGTCGGGAGGCTGATTTTCTCTGGTGAGGCCCTTACCTA
CAAAGACATTGTAGTGTGCATGGATGGAGACACCATGCCTGGCCTCTTTGCCTACAGAGCCG
CCACCAAGGCTGGCTACTGTGGAGGAGCCGTTCTCGCTAAGGACGGAGCTGACACATTCATC
GTTGGCACTCACTCCGCAGGTGGTAACGGAGTTGGGTACTGCTCGTGCGTATCCAGGTCCAT
GCTTCTGAAAATGAAGGCACACATCGACCCCGAACCACACCATCAGTAG

AcGFP

ATGGTGAGCAAGGGCGCCGAGCTGTTCACCGGCATCGTGCCCATCCTGATCGAGCTGAATG
GCGATGTGAATGGCCACAAGTTCAGCGTGAGCGGCGAGGGCGAGGGCGATGCCACCTACGG
CAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCTGTGCCCTGGCCCACCCTG
GTGACCACCCTGAGCTACGGCGTGCAGTGCTTCTCACGCTACCCCGATCACATGAAGCAGC
ACGACTTCTTCAAGAGCGCCATGCCTGAGGGCTACATCCAGGAGCGCACCATCTTCTTCGA
GGATGACGGCAACTACAAGTCGCGCGCCGAGGTGAAGTTCGAGGGCGATACCCTGGTGAAT
CGCATCGAGCTGACCGGCACCGATTTCAAGGAGGATGGCAACATCCTGGGCAATAAGATGG
AGTACAACTACAACGCCCACAATGTGTACATCATGACCGACAAGGCCAAGAATGGCATCAA
GGTGAACTTCAAGATCCGCCACAACATCGAGGATGGCAGCGTGCAGCTGGCCGACCACTAC
CAGCAGAATACCCCCATCGGCGATGGCCCTGTGCTGCTGCCCGATAACCACTACCTGTCCA
CCCAGAGCGCCCTGTCCAAGGACCCCAACGAGAAGCGCGATCACATGATCTACTTCGGCTT
CGTGACCGCCGCCGCCATCACCCACGGCATGGATGAGCTGTACAAGTGA

Table 3

Nucleotide variations in the IRES motifs of FMDV and EMCV sequence

MOTIF	FMDV-O-IRES KP019381	FMDV-A-IRES KP019382	FMDV-Asia1-IRES KP019383	EMCV IRES
GNRA motif	GCAA	GUAA	GCAA	GCGA
Hexaloop	AACCCC	AUUCCC	AAUCCC	AACCCC
Apical stem	CGAAA	CGAAA	CGAAA	CAAAA
C-Rich Motif	AACCCCA	AACCCCA	AACCCCA	AACCCCA
Pyrimidine rich apical loop	UCUUU	UCUUU	GCUUU	UCUUU

```
O-IRES      AGCAGGTTTCCCCAACTGACACAAACCGTGCAACTTGAAACTCCGCCTGGTCTTTCCAGG 60
A-IRES      AGCAGGTTTCCCCAACTGACACACACCGTACAATTTGAAACTCCGCCTGGTCTTTCCAGG 60
As-IRES     TGCAGGTAGCCCCAACTGACACAAACCGTGCAACTTGGAACCCCGCCTGGGCTTTCCAGG 60
            :******: **************.*****.*** ***.*** ******** *********

O-IRES      TCTAGAGGGGTGACATTTTGTACTGTGCTTGACTCCACGCTCGGTCCACTGGCGGGTGCT 120
A-IRES      TCTAGAGGGGCGACACTTTGTACTGTGATTGACTCCACGCTCGGTCCACTGGCGGGTGCG 120
As-IRES     TCTAGAGGGGTGACGCCTTGTACTGTGTTTGACTCCACGCTCGGTCCACTAGCGAGTGTT 120
            ********** ***.  ********** **********************.***.***

O-IRES      AGTAGTAGCACTGTTGCTTCGTAGCGGAGCATGATGGCCGTGGGAACCCCTCCTTGGCAA 180
A-IRES      AGTAACAGGACTGTTGCTTCGTAGCGGAGCGTGTTGGCCGTGGGATTCCCTCCTTGGTAA 180
As-IRES     AGTAGTAGTACTGTTGCTTCGTAGCGGAGCATGACGGCCGTGGGAATCCCTCCTTGGCAA 180
            ****. ** *********************.**: **********: ********** **

O-IRES      CAAGGACCCACGAGGCCGAAAGCCACGTCCTAACGGACCCATCATGTGTGCAACCCCAGC 240
A-IRES      CAAGGACCCACGGGGCCGAAAGCCACGTCCTAACGGACCCAACATGTGTGCAACCCCAGC 240
As-IRES     CAAGGACCCACGGGGCCGAAAGCCACGTCCTGAAGGACCCGTCATGTGTGCAACCCCAGC 240
            ************.******************.*.******.:******************

O-IRES      ACGGCAACTTTACTGTGAAAACCACTTCAAGGTGACACTGATACTGGTACTCAAACACTG 300
A-IRES      ACGGCAACTTTACTGTGAAAACCACTTTAAGGTGACACTGATACTGGTACTCAACCACTG 300
As-IRES     ACGGCAGCTTTATTATGAAACCCACTTTAAGGTGACACTGATACTGGTACTCAAACACTG 300
            ******.***** *.*****.****** **************************.*****

O-IRES      GTGACAGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGAGACACTCGGGATCTGA 360
A-IRES      GTGACAGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGCGACACTCGGGATCTGA 360
As-IRES     GTGACAGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGCGACACTCGGGATCTGA 360
            ******************************************.*****************

O-IRES      GAAGGGGACTGGGGCTTCTTTAAAAGTGCCCAGTTTGAAAAGCTTCTACGCCTGAATAGG 420
A-IRES      GAAGGGGACTGGGGCTTCTTTAAAG-CGCCCAGTTTAAAAAGCTTCTATGCCTGAATAGG 419
As-IRES     GAAGGGGACTGGGGCTTCTATAAAAGTGCCCAGTTTAAAAAGCTTCTATGCCTGGATAGG 420
            *******************:****.  *********.*********** *****.*****

O-IRES      CGACCGGAGGCCGGCGCCTTTCCATTAATTACTACTACTTTCA 463
A-IRES      CGACCGGAGGCCGGCGCCTTT-CTTTACCATTACTAAACTG- 460
As-IRES     CGACCGGAGGCCGGCGCCTTTCCTTTGACCACTACTGTTTAC- 462
            ********************* *:** *  * ****. : :
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Fig. 8. Multiple Sequence alignment of FMDV IRES sequence of Indian vaccine strains.

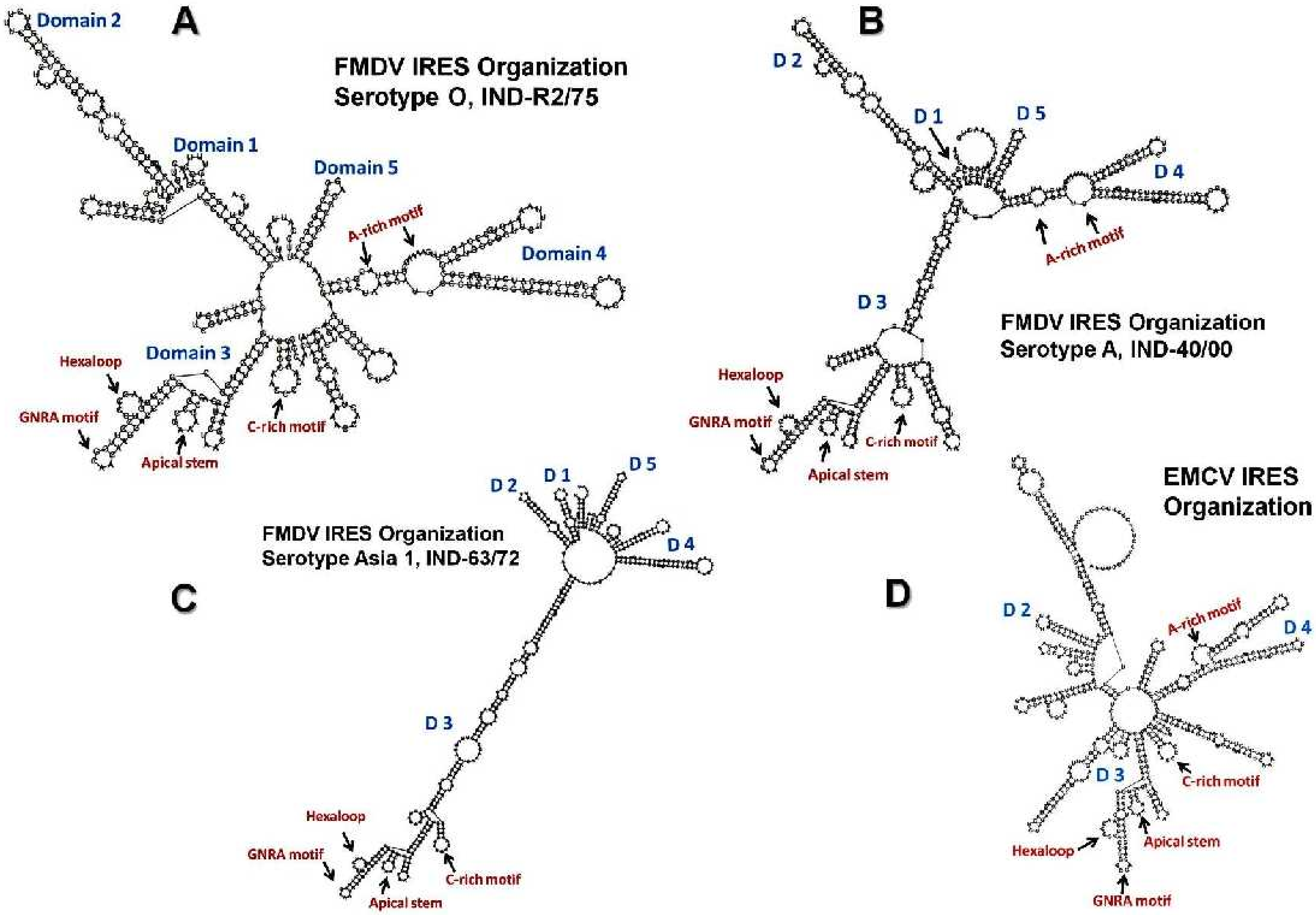


Fig. 9 . Secondary structures organization prediction of FMDV (3 serotypes) and EMCV IRES. Conserved motifs and different domains has been labelled. D1 – D5: Domain 1 – Domain 5.

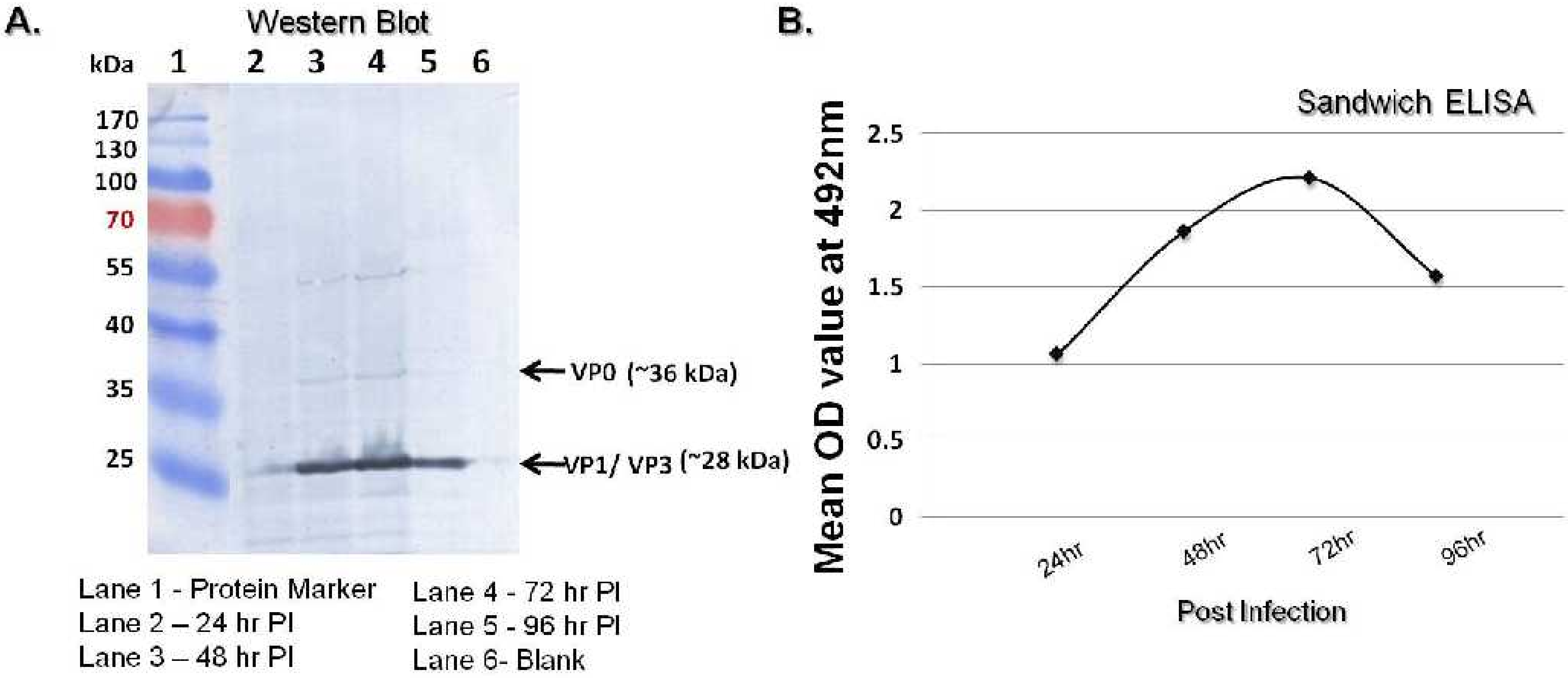


Fig. 10. Determination of the expression kinetics of recombinant capsid protein in Tn-5 insect cells at different time interval by **A.** Western Blot and **B.** Sandwich ELISA. The maximum expression of capsid protein was observed at 72 hours post-infection (PI).

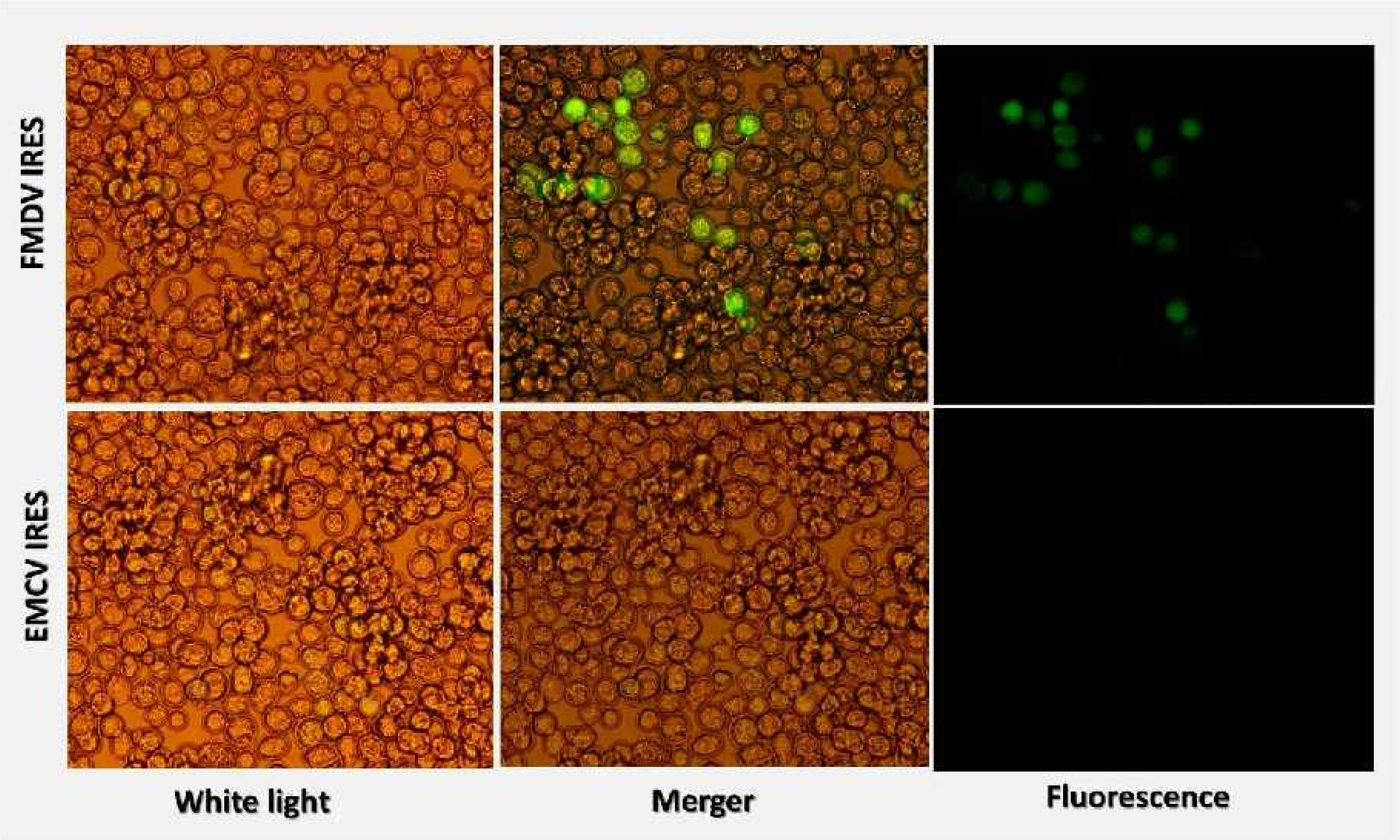
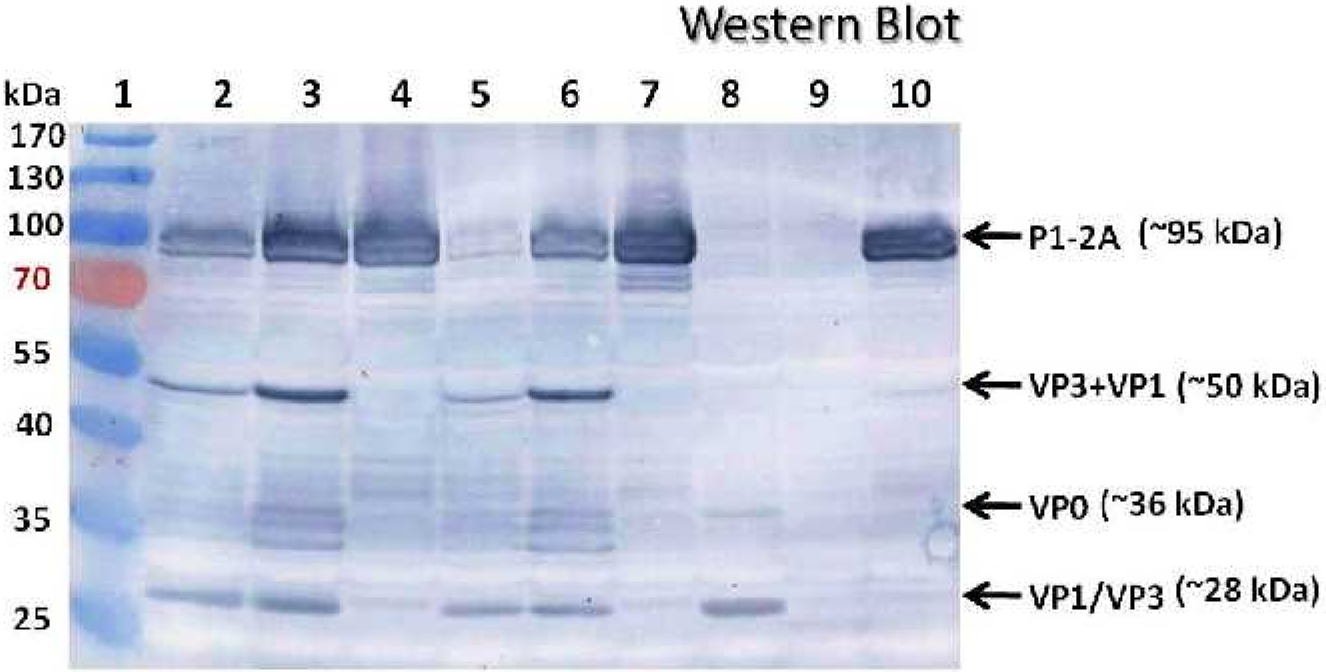
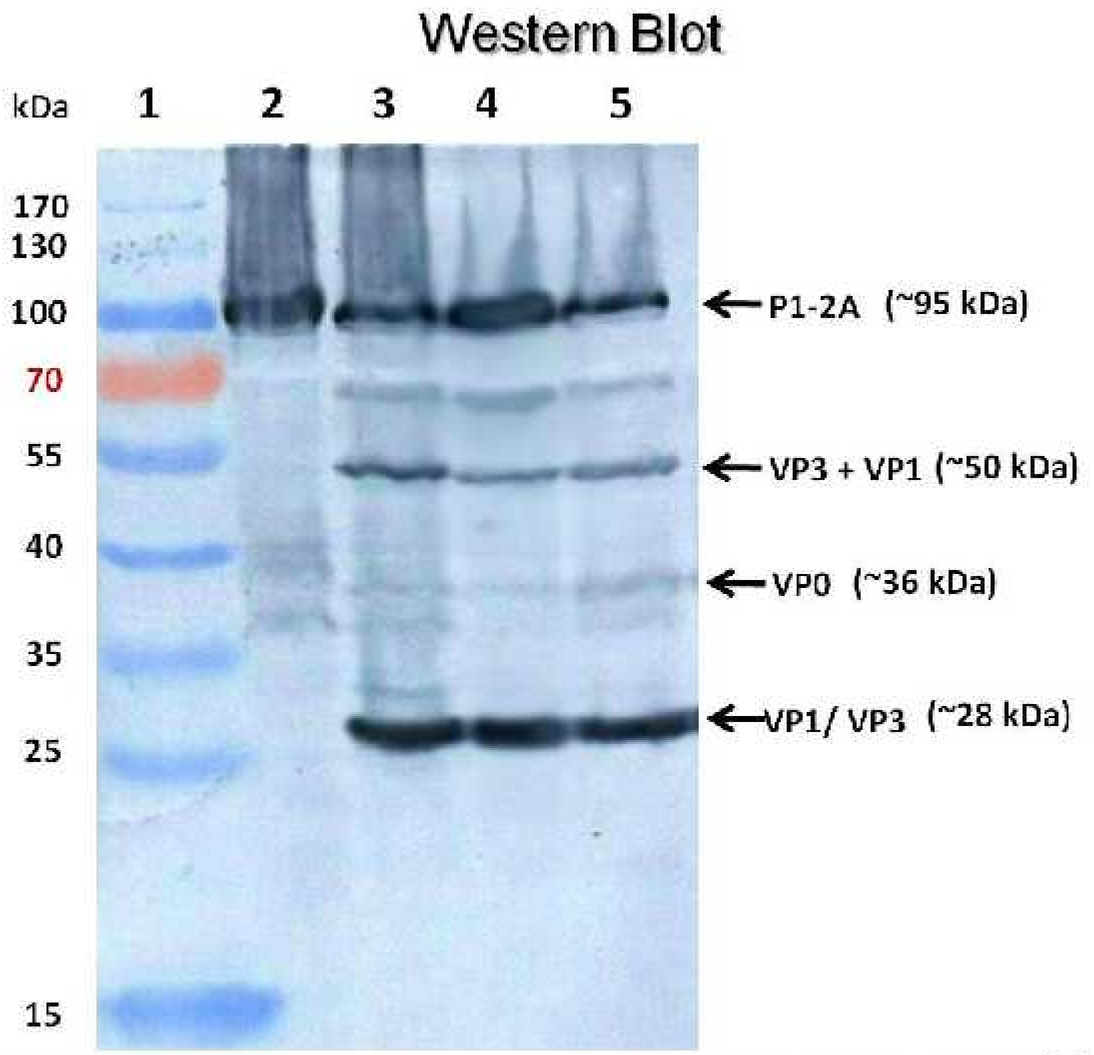


Fig. 11. Expression of green fluorescent protein (GFP) observed under florescent microscopy (X 400). Expression of GFP was evident in the construct containing FMDV P1-2A and AcGFP separate by FMDV IRES (Upper panel) while no fluorescence was detectable in similar construct containing EMCV IRES (lower panel).



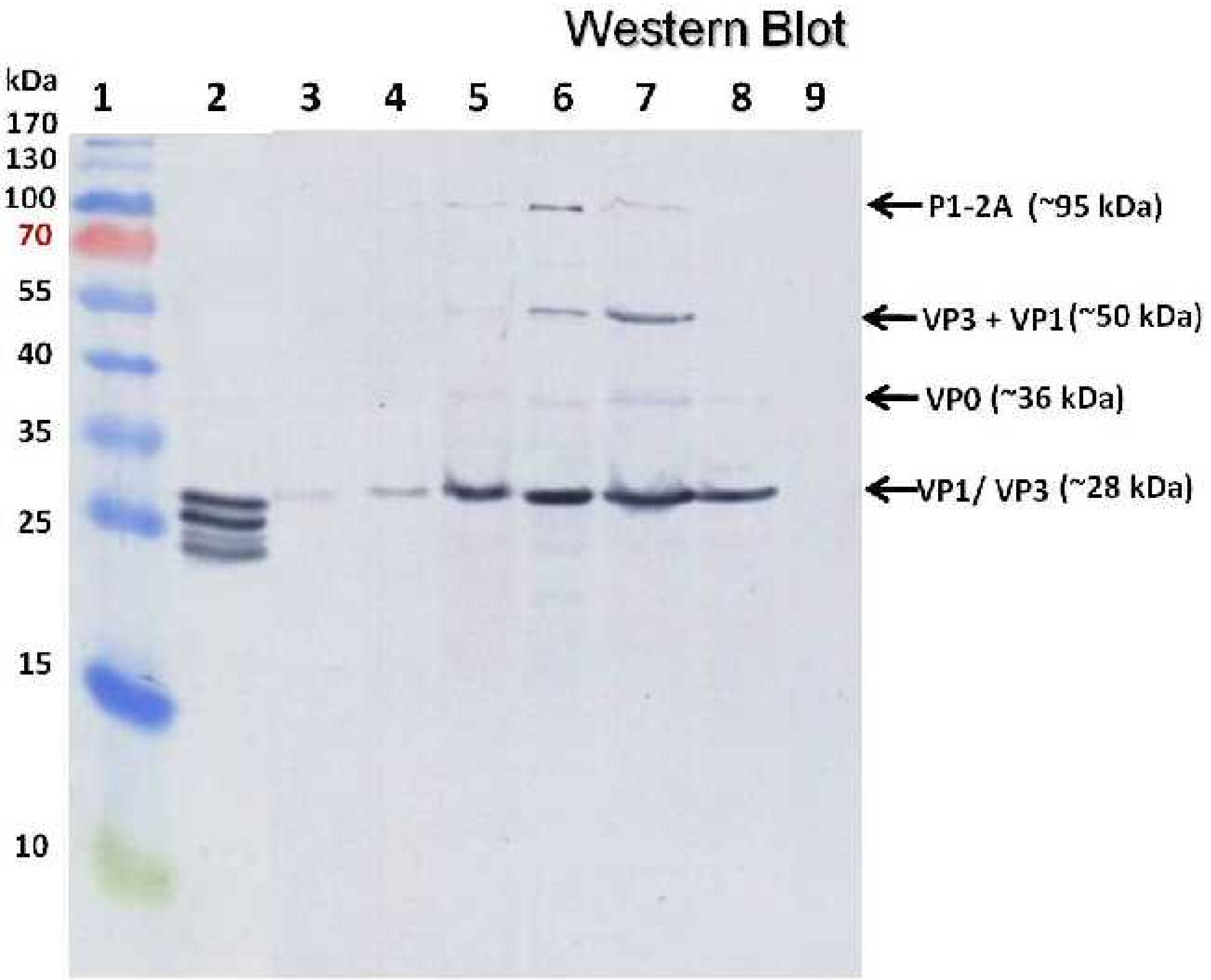
Lane 1 - Protein Marker
Lane 2 - pFB-P12A-FMDV_O_IRES-3Cpro^{wt}
Lane 3 - pFB-P12A-FMDV_O_IRES-3Cpro^{mut}
Lane 4 - pFB-P12A-FMDV_O_IRES-AcGFP
Lane 5 - pFB-P12A-FMDV_As1_IRES-3Cpro^{wt}
Lane 6 - pFB-P12A-FMDV_As1_IRES-3Cpro^{mut}
Lane 7 - pFB-P12A-FMDV_As1_IRES-AcGFP
Lane 8 - pFD-PH-P12A / P10-P12A-3Cpro^{wt}
Lane 9 - pFD-PH-P12A / P10-3Cpro^{wt}
Lane 10 - pFB-P12A-EMCV_IRES-3Cpro^{wt}

Fig. 12. Expression screening of different recombinant cDNA cassettes used in this study by immunoblotting using a polyvalent O serum. The expected migration positions of P1-2A (~95kDa), VP1+VP3 (~50kDa), VP0 (~36kDa) and VP1/VP3 (~28kDa) are indicated at right. Numbers to the left are the migration positions of protein markers



Lane 1 - Protein Marker
Lane 2 - pFB-P12A-EMCV IRES-3Cpro^{wt}
Lane 3 - pFB-P12A-FMDV_O_IRES-3Cpro^{wt}
Lane 4 - pFB-P12A-FMDV_A_IRES-3Cpro^{wt}
Lane 5 - pFB-P12A-FMDV_Asia1_IRES-3Cpro^{wt}

Fig. 13. Expression screening of FMDV P12A-IRES-3C cDNA cassettes by immunoblotting using a polyvalent O serum. Expression cassette containing EMCV IRES failed to process the structural polyprotein (P12A). No obvious difference noticed between IRES element of three FMD serotypes in polyprotein processing. The expected migration positions of P1-2A (~95kDa), VP1+VP3 (~50kDa), VP0 (~36kDa) and VP1/VP3 (~28kDa) are indicated. Numbers to the left are the migration positions of protein markers.



Lane 1 - Protein Marker
Lane 2 - FMDV- O-146S Ag
Lane 3 - pFB-P1-2A-3Cpro^{wt}
Lane 4 - pCMV-P12A-FMDV_A_IRES-3Cpro^{wt}
Lane 5 - pFB-P12A-FMDV_A_IRES-3Cpro^{wt}
Lane 6 - pFB-P12A-FMDV_A_IRES-3Cpro^{mut}
Lane 7 - pFD-PH-P12A / P10-P12A-3Cpro^{mut}
Lane 8 - pFD-PH-P12A / P10-P12A-3Cpro^{wt}
Lane 9 - pFD-PH-P12A / P10-3Cpro^{wt}

Fig. 14. Expression screening of different recombinant cDNA cassettes used in this study by immunoblotting using a polyvalent O serum. The expected migration positions of P1-2A (~95kDa), VP1+VP3 (~50kDa), VP0 (~36kDa) and VP1/VP3 (~28kDa) are indicated. Numbers to the left are the migration positions of protein markers.

Three constructs containing IRES derived from all the three serotypes behaved similarly and showed almost identical efficiency when infected at same MOI in insect cells (Fig 13, Lane 3, 4, 5).

Construct carrying polyprotein (P12A) under polyhedrin promoter and GFP under EMCV IRES (construct 4) yielded no detectable GFP expression (Fig. 11) or no cleavage of the capsid polyprotein (~95 kDa) with the construct having 3Cpro^{wt} under EMCV IRES (construct 5) as confirmed by the immunoblot (Fig. 12, Lane 10; Fig. 13, Lane 2).

Next strategy was attempted with the dual vector system with the feature of the expression designed here, which differs from previous construct, is that two transcription unit (construct 6, 7, 8) driven by the Ph and P10 promoter bi-directionally. Dual vector expression of processed viral capsids having 3C along with P1 showed interesting results. It was observed that expression of P1-2A and 3Cpro^{wt} as two ORFs under the control of two independent promoters (construct 6) did not gave the desired results. In the lysate from this construct, no processed capsids were seen in the western blot (Fig. 12, Lane 9; Fig. 14, Lane 9), while expected bands of processed capsids were obtained in the other dual promoter constructs containing two copy number P1-2A with single 3Cpro (Fig. 12, Lane 8; Fig. 14, Lane 8). Construct expressing P1-2A under Ph promoter and P1-2A-3Cpro^{wt} under P10 promoter (Construct 7) gave a complete capsid polyprotein processing and gave VP1/VP3 band of higher intensity than the other constructs in this experiment (Fig. 12, Lane 8). Results of 3Cpro^{mut} (in place of 3Cpro^{wt}) (construct 8) cleavage was comparable and had less proteolytic cleavage of P1-2A than 3Cpro^{wt} (i.e) presence of P12A polyprotein and VP3+VP1 intermediate precursor protein band (Fig. 14, Lane 8).

The unidirectional-monocistronic P1-2A-3C construct expressing both genes as single transcriptional unit driven by Polyhedrin promoter (construct 9) showed the lowest yield of VP1/VP3 protein with other baculoviral constructs (Fig. 14, Lane 3).

4.5.1. Sandwich ELISA

Results of sandwich ELISA and western blot were comparable (Fig. 15). There was difference between the signals detected using the intact precursor and the processed products. The unprocessed P1-2A precursor i.e. intact polyprotein or incompletely processed P12A reacted well in diagnostic serotype O specific antigen ELISA and showed a higher OD signal by sandwich ELISA in comparison with the inactivated FMD O antigen. Among the processed polyprotein, the construct carrying two copy number of P1-2A polyprotein with single 3Cpro^{wt} (Construct 7) showed a good immunoreactivity in comparison with the other constructs. Among all constructs, the cassette expressing P1-2A and 3C as two separate ORFs under the control of two independent promoters (construct 6) showed lower reactivity with anti-serum in s-ELISA.

4.6. Generation of recombinant adenovirus plasmid clone

The constructed recombinant pShuttle-CMV plasmid carrying P12A-FMD_A_IRES-3C were linearized using *Pme I* restriction enzyme and then transformed to chemically competent BJ5183-AD-1 cells for homologous recombination at left and right arm with pAdEasy-1 vector to generate recombinant adenovirus plasmids (pAd-P12A-AIRES-3C). The recombinant pAdEasy-1 plasmid isolation were analysed for the insert by PCR using gene specific primers as well as by *Hind III* restriction enzyme site digestion and release of multiple fragments were analysed by 1% agarose gel electrophoresis with respect to agarose gel simulate using SnapGene version 2.5 software (Fig. 16).

4.7. Generation of recombinant adenovirus and expression of FMDV capsid proteins in HEK-293 cells

These recombinant adenovirus plasmids were digested with *PacI* enzyme to expose the inverted terminal repeats (ITR) and transfected into 50% confluency HEK-293 cells cultured in 6 well tissue culture plate using Virapack Transfection Kit. The transfected cells were observed daily upto 10 days for the appearance of virus induced plaques. Plaques observed 5th day post transfection were individually picked up carefully followed by centrifuging and re-suspended in Dulbecco's phosphate buffer saline (DPBS) containing Ca++ and Mg++ and freeze-thawed thrice to release the virus. The supernatant containing the virus were titrated and the viruses having the higher were selected to prepare seed viruses. These recombinant seed virus were propagated 2-3 rounds to amplify the viral titers in HEK cells. Recombinant viruses were used at 0.1 MOI to amplify the virus titer and they were amplified to high titers ($1x10^9$ pfu/ml) by 2nd or 3rd passage in HEK cells.

The recombinant adenovirus at 5 MOI were infected the HEK cells grown in 175

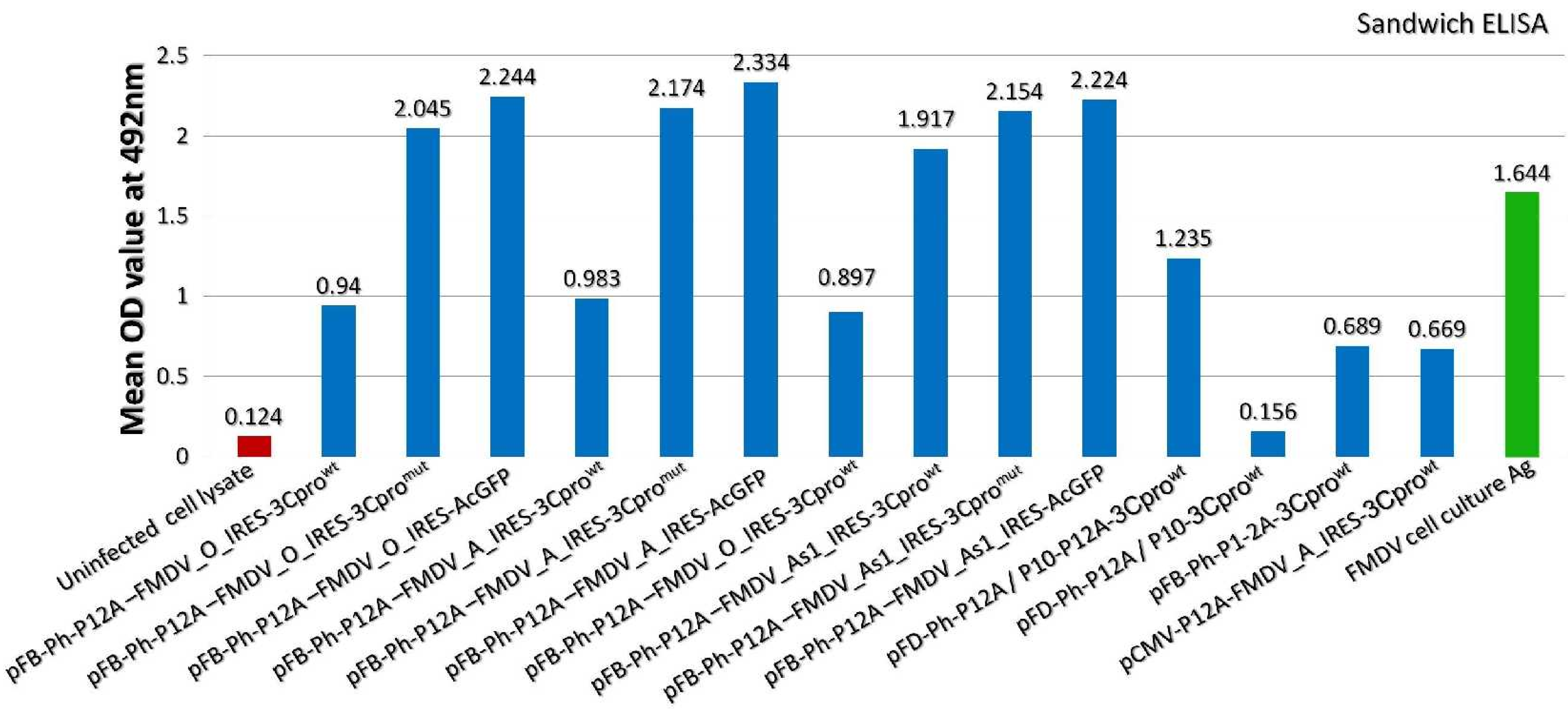
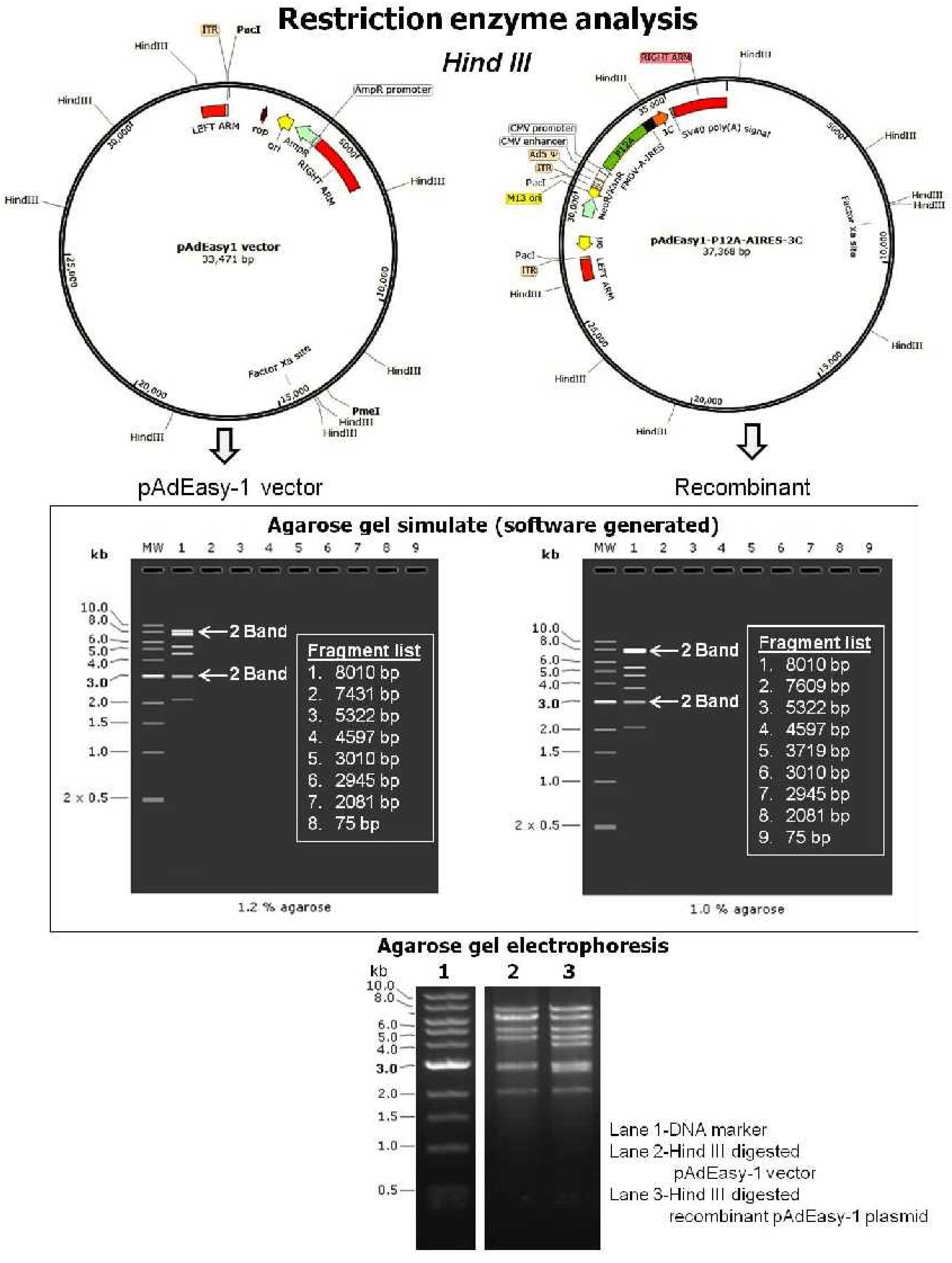


Fig. 15. Sandwich ELISA of the expressed FMDV capsid proteins obtained from the different cDNA cassettes in this study using O serotype-specific anti-FMDV antibodies. Processed serotype O P1-2A protein is less recognized by the rabbit anti-FMDV antisera used for antigen capture than the intact proteins (unprocessed).



Fragment list
1. 8010 bp
2. 7431 bp
3. 5322 bp
4. 4597 bp
5. 3010 bp
6. 2945 bp
7. 2081 bp
8. 75 bp

Fragment list
1. 8010 bp
2. 7609 bp
3. 5322 bp
4. 4597 bp
5. 3719 bp
6. 3010 bp
7. 2945 bp
8. 2081 bp
9. 75 bp

Lane 1-DNA marker
Lane 2-Hind III digested pAdEasy-1 vector
Lane 3-Hind III digested recombinant pAdEasy-1 plasmid

Fig. 16. Restriction enzyme analysis of the recombinant pAdEasy-1 plasmid containing P12A-FMD_A_IRES-3Cpro^{wt} using Hind III enzyme.

cm2 culture flask as monolayer and incubated at 37 °C for 3 days. Infected cells were then recovered after 72 hour post infection by pelleting and lysed in lysis buffer containing 0.1% Triton X 100. The lysate obtained were homogenized by syringe followed by centrifugation and the supernatant collected were subjected for expression analysis by western blot and sandwich ELISA.

4.8. Characterization of the recombinant adenoviral clone expressing FMDV capsid protein

The bicistronic construct carrying P12A-FMD_A_IRES-3Cpro^{wt} driven by CMV promoter in adenoviral expression system expressing 3Cpro^{wt} under FMDV IRES (construct 10) showed low yield of VP1/VP3 protein in comparison with baculoviral expression system (Fig. 14, Lane 4). Results of sandwich ELISA and western blot were comparable and it was observed a low immunoreactivity with the antisera raised against FMD type O antigen (Fig. 15).

4.9. Purification of FMDV type O VLPs expressed in insect cells

For purification of the empty capsid, infected Tn-5 cells at 5 MOI were collected by centrifugation on 3 days post infection followed by pelleting and re-suspended in lysis buffer. The re-suspended lysate were sonicated and the clarified supernatant was over layered onto 30% sucrose cushion and ultra-centrifuged. This cushioned pellet was suspended in Tris-NaCl buffer overnight (4ºC) and applied to a discontinuous 30–60% sucrose gradient with the increment of 10% of four gradients in polyallomer tubes. After ultracentrifugation, the fractions were collected (Fig. 17) and the reactivity of each fraction was checked for antigenicity by comparing with inactivated BHK-21 grown FMDV antigen, in western blot and sandwich ELISA. The results shows that empty capsid accumulate in the sucrose density of 45-50% as evident from the western blot and sandwich ELISA (Fig. 18 and 19). The fractions from the middle of the gradient (at 45-50% sucrose level), had good immunoreactivity signal as seen in sandwich ELISA and they showed bands corresponding to VP1/VP3 subunit in immunoblot assay (Fig. 18 and 19). The fractions collected were also were tested for spectrophotometry followed by estimation of whole protein concentration (mg/ml) as per the formula [1.55 x A280]–[0.76 x A260]. Similarly, the fractions from the middle of the gradient i.e. 50% sucrose level had maximum concentration of 2mg/ml of purified fraction (Fig. 20) (Table 4).

4.10. VLP yield: quantitative analysis

The baculoviral expressed VLP samples of three different constructs (construct 2, 7, 9) (Fig. 2) has been quantified by sandwich ELISA (Basagoudnavar et al., 2015). In this experiment, similar conditions were followed in all the levels of VLP expression (infection, protein processing and purification) for all the three constructs mentioned above. In Tn-5 cells, P12A-P12A-3Cpro^{wt} (construct 7) showed ~8 fold and ~64-fold better VLP yield than P12A-IRES-3Cpro^{wt} (construct 2) and P12A3C construct (construct 9), respectively (Fig. 21 and Table 5). Similarly, P12A-IRES-3Cpro^{wt} (construct 2) showed ~8 fold better VLP yield than P12A3C construct (construct 9).

4.11. Transmissible Electron Microscopy (TEM)

The peak purified FMD O immunoreactive fraction stained by 1% uranyl acetate revealed authentic structure typical of picornaviral capsids of about ~25nm size diameter under electron microscopy (Fig. 22).

4.12. Purification and quantification of inactivated concentrated FMDV virus

Purification was done by CsCl density gradient and the 146S was quantified spectrophotometrically. Six fractions for each serotype were collected and the absorbance of the fractions was measured at 259 and 239 nm. The fraction with maximum absorbance at 259 and minimum at 239 with a ratio of 1.3-1.4 at 259/239 were selected and 146 ‘S’ content was estimated with an extinction coefficient (E1% 259). The fractions containing 146 ‘S’ were pooled and dialysed extensively overnight against Tris-NaCl buffer at 4˚C to remove CsCl salt.

4.13. Guinea pig experiment

The immunization study of the different FMD vaccine formulations was done in guinea pigs. No untoward effects were recorded in the guinea pigs following immunization with any of the formulations.

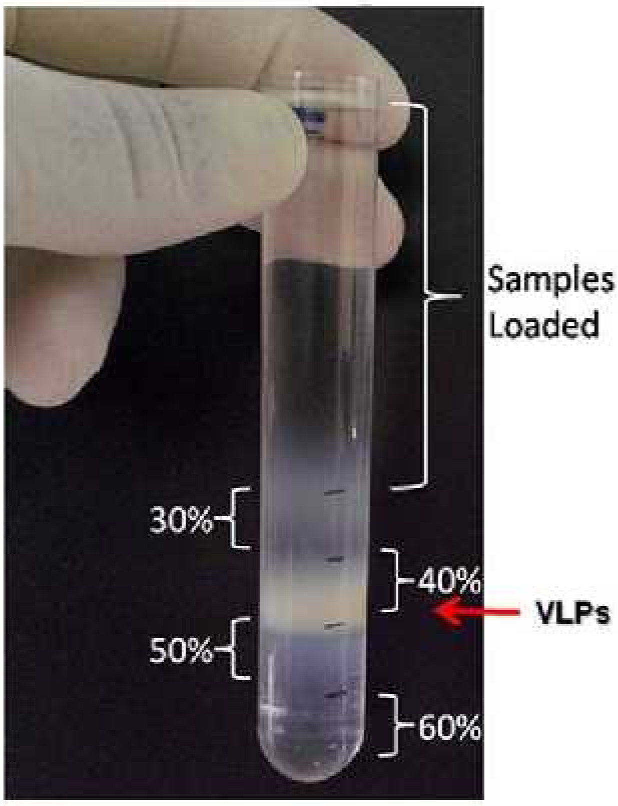


Fig. 17. Photographic Image: After ultracentrifugation of expressed VLP lysate in the discontinuous 30–60% sucrose gradient with the increment of 10% of four gradients in 17ml polyallomer tubes.

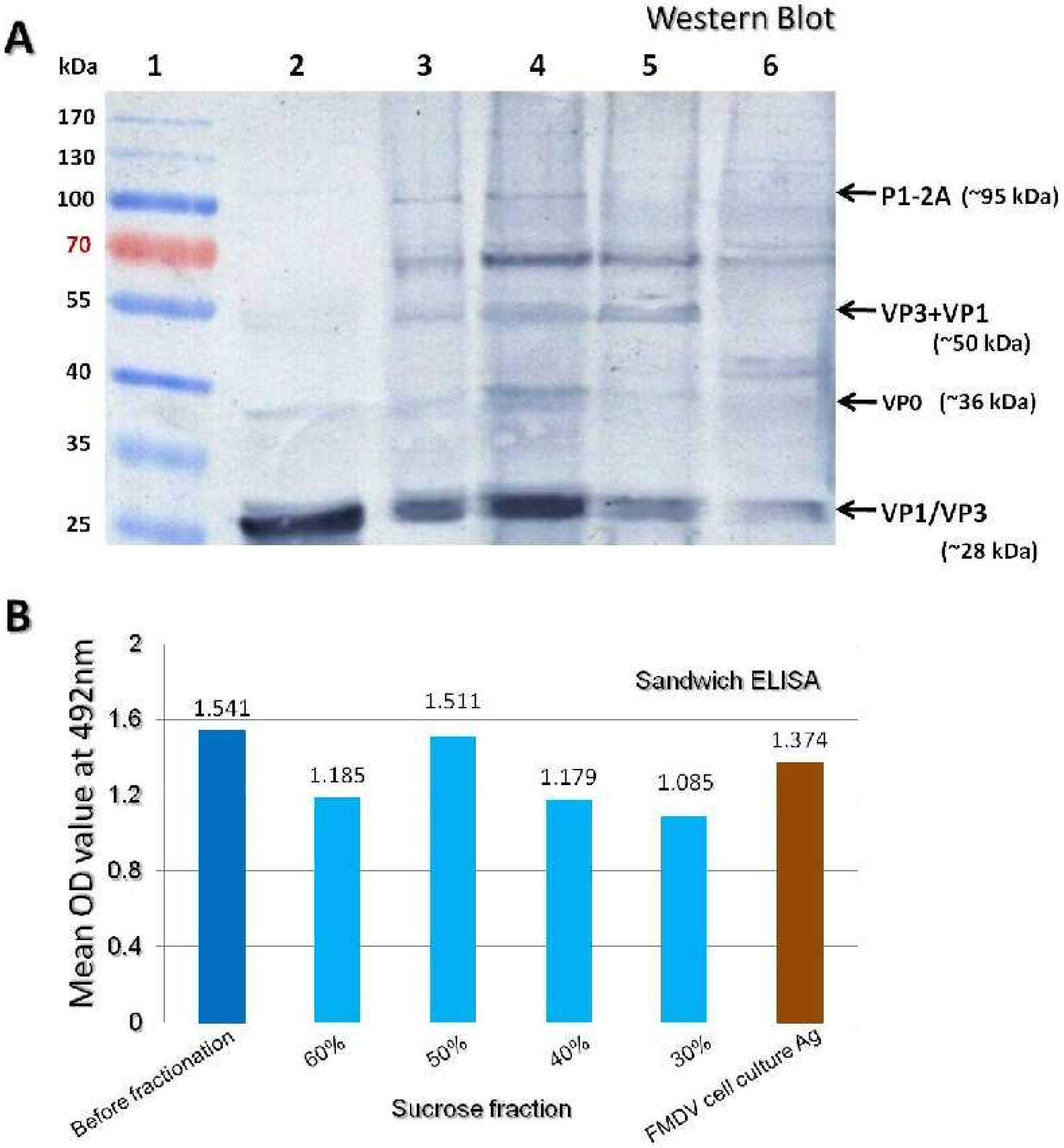


Fig. 18. A. Western blot analysis of sucrose gradient fractions of FMDV capsid probed with rabbit sera raised against FMDV type O (Ind-R2/75). Lane 1-Protein Marker, Lane 2- BHK-21 grown FMDV antigen, Lane 3 to 6- Fractions collected from 60%, 50%, 40% and 30% sucrose fractions. **B.** Sandwich ELISA of purified FMD Virus like particles from various sucrose gradient fractions with anti-146S FMDV serum.

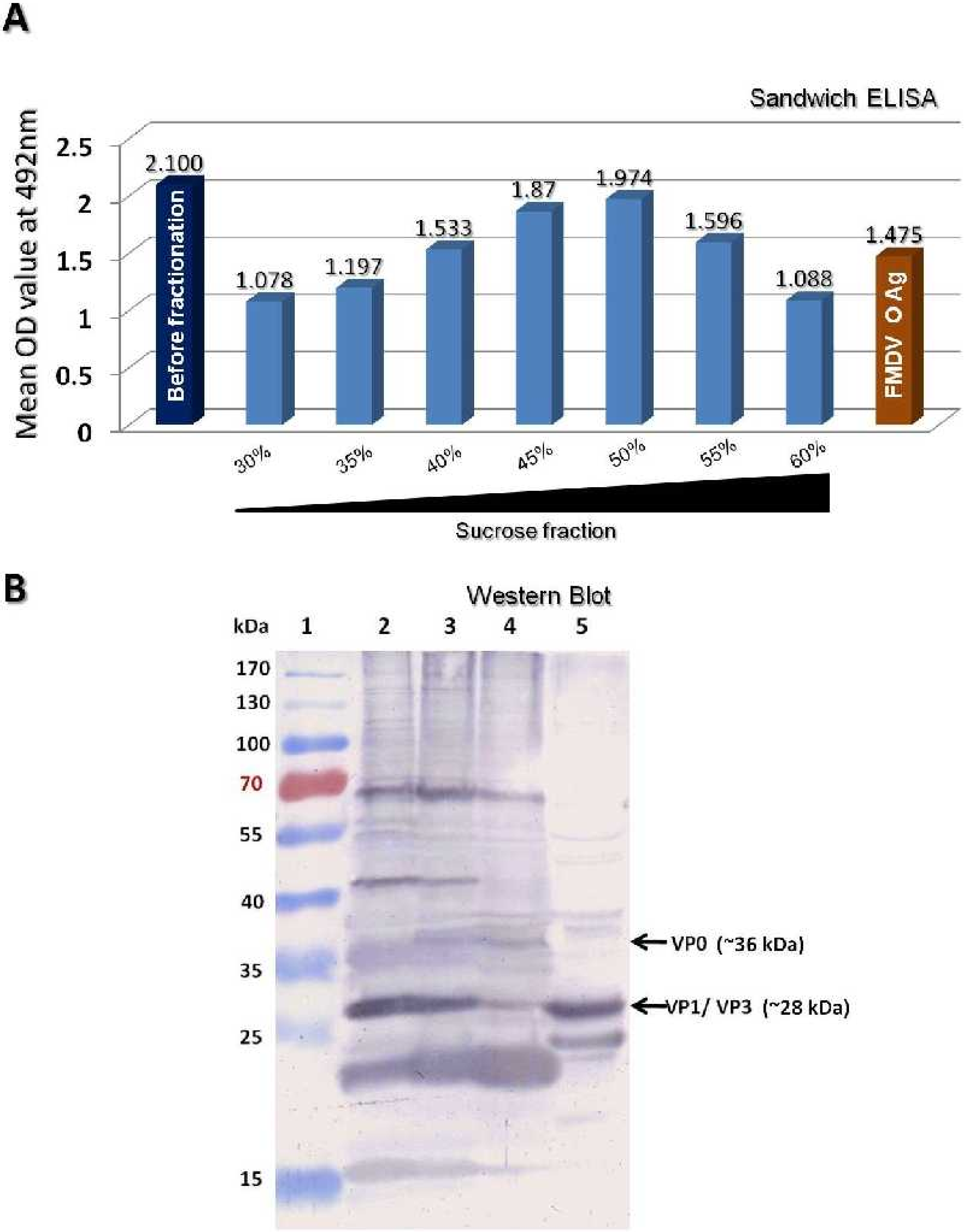


Fig. 19. A. Sandwich ELISA of purified FMD Virus like particles after ultracentrifugation of the discontinuous 30–60% sucrose gradient with the increment of 5% of seven gradient in 17ml polyallomer tubes with anti-146S FMDV serum. **B.** Western blot analysis of sucrose gradient fractions of FMDV capsid probed with rabbit sera raised against FMDV type O (Ind-R2/75). Lane 1- Protein Marker, Lane 2 to 4- Fractions collected from 50%, 45% and 40% sucrose fractions, Lane 5- BHK-21 grown FMDV antigen.

Table 4

Spectrophotometry reading and total protein concentration of various sucrose gradient fractions after ultracentrifugation

FRACTION	A260 nm	A280 nm	Total protein concentration (mg/ml)
60% Sucrose	2.499	2.349	1.74
50% Sucrose	2.747	2.652	2.02
40% Sucrose	2.596	2.455	1.83
30% Sucrose	2.597	2.472	1.86

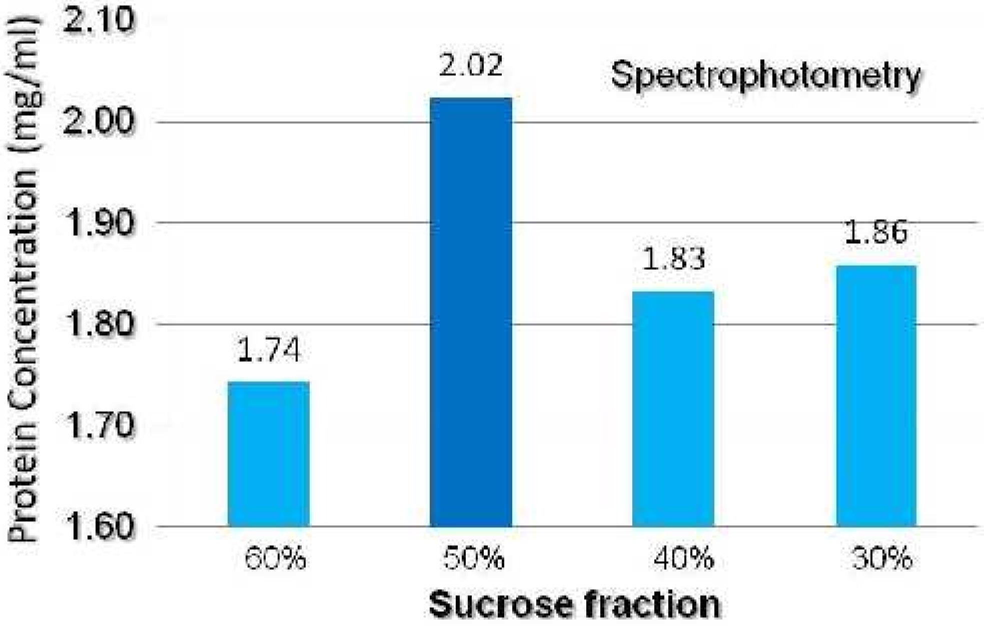


Fig. 20. Protein concentration of various sucrose gradient fractions after ultracentifugation.

Table 5
FMD VLP quantitative analysis of expressed VLPs from three different constructs by sandwich ELISA

Sample (µl)	Fold dilution	FMD-O-Ag Concentration (µg)	FMD-O-Ag	P1-2A-3C	P12A-A_IRES-3C	P12A-P12A-3C
			Mean OD at 492nm (Sandwich ELISA)			
50	1	10	1.3589	0.46125	1.00385	1.16565
25	2	5	1.27	0.33895	0.85755	1.13785
12.5	4	2.5	1.17575	0.29715	0.7468	1.03565
6.25	8	1.25	1.15595	0.15375	0.51925	0.9592
3.125	16	0.625	1.0235	0.10345	0.323	0.84625
1.5625	32	0.3125	0.94635	0.0717	0.22445	0.62505
0.78125	64	0.15625	0.87825	0.063	0.15955	0.51215
0.390625	128	0.078125	0.7735	0.05405	0.10785	0.39555
0.1953125	256	0.0390625	0.6385	0.05245	0.0825	0.2334
0.09765625	512	0.0195313	0.4868	0.04845	0.0647	0.15045
0.048828125	1024	0.0097656	0.3546	0.04835	0.0512	0.1157

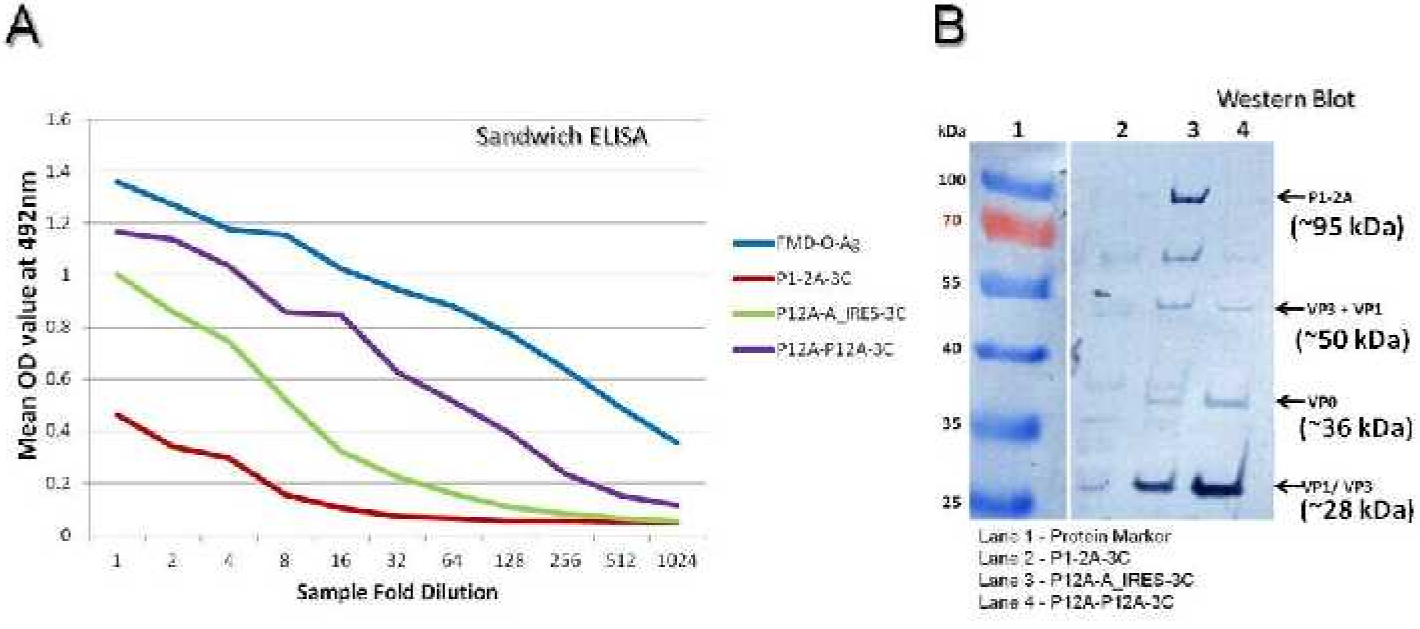


Fig. 21. A. Graphical representation: FMD VLP quantitative analysis of expressed VLPs from three different constructs along with the known FMDV 'O' antigen by sandwich ELISA. **B.** Western blot analysis of these expressed VLPs for FMDV capsid probed with rabbit sera raised against FMDV type O (Ind-R2/75).

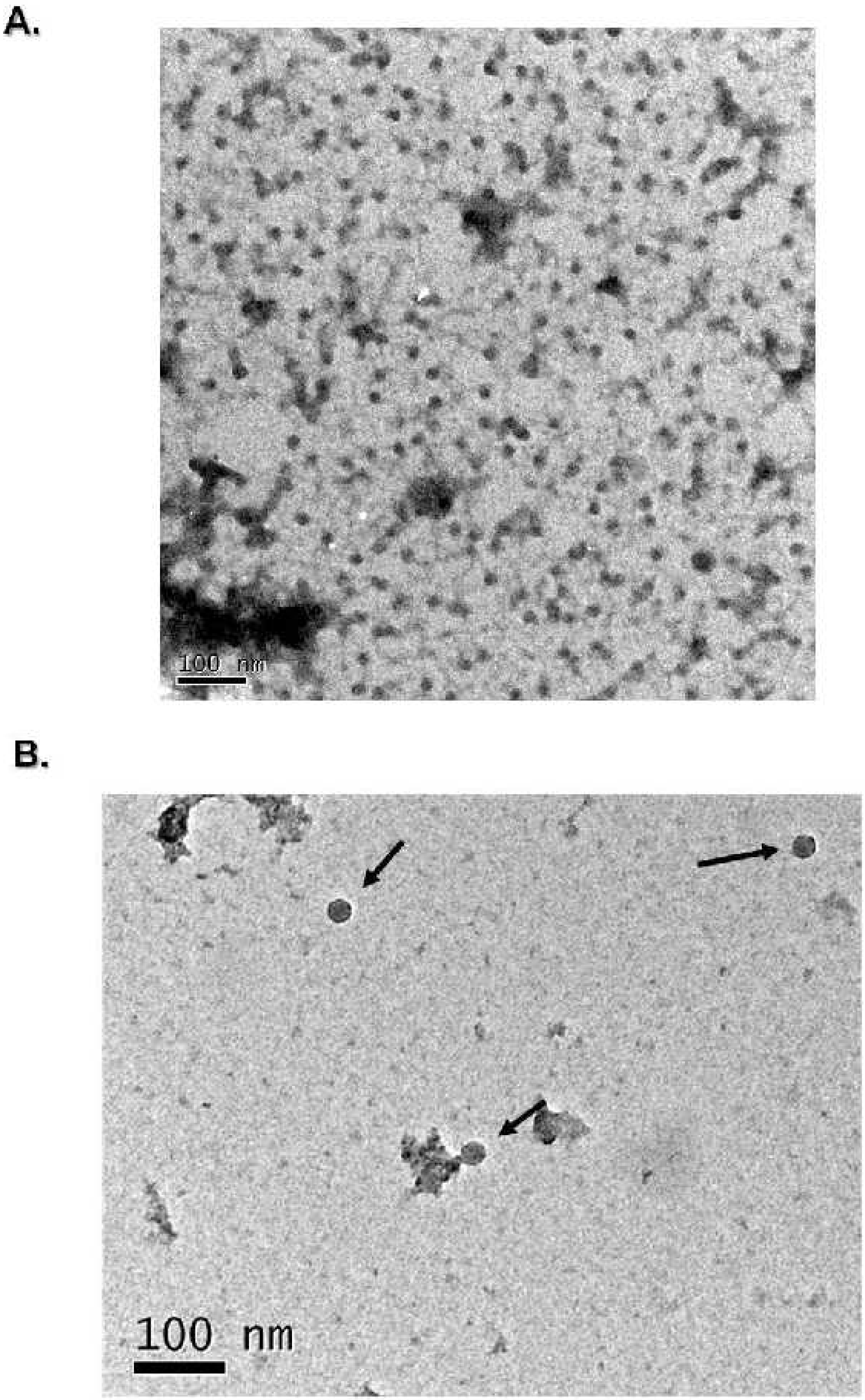


Fig. 22. Electron Micrograph. Analysis of empty capsid assembly by TEM stained by 1% uranyl acetate. Empty capsid was synthesized from pFB-P12A-FMDV_A_ IRES-3Cpro^{wt} construct for TEM imaging. Typical structure of picornaviral spherical capsids of about size ~25nm diameter under transmission electron microscopy was noticed. The bar indicates 100nm size.

4.13.1. Virus neutralization test

This test was performed to assess the humoral immune response in terms of virus neutralising antibodies against FMDV serotype O on day 0, 14, 28, 42 and 56 days post immunization intramuscularly in the experimental groups administered with different formulations of vaccines. The virus neutralizing antibody titres were expressed in terms of mean $\log_{10}$ serum neutralizing antibody_{50}/100 TCID50 of virus. On the day of immunization, all the guinea pigs used were negative to FMDV specific antibodies. All the animals in each group immunized with different vaccine formulations showed antibody production (Fig. 23; Table 6). The details of neutralizing antibody titre against each serotype with different vaccines formulations are indicated in (Fig. 23; Table 6). The highest mean $\log_{10}$ SN_{50} against FMDV type O by the animals in Group III administered with conventional vaccine was 2.08 ± 0.09 (mean ± SE). This titre was observed on 56th day post vaccination. Similarly, Group II (VLPs) animals exhibited highest mean $\log_{10}$ SN_{50} against FMDV type O on 56th days post vaccination with titre of 1.40 ± 0.09. Group I animals did not show any antibody response following PBS administration (as placebo) as expected. When the groups were compared using Student's t-test for the production of neutralizing antibodies over a period of 56 days post vaccination, there was a no significant difference between Group II and III ($t = 0.063$, $P>0.05$).

4.13.2. Indirect ELISA

Indirect ELISA showed that the specific antibody response of guinea pigs vaccinated with inactivated whole virus vaccine increased greatly during the 28 dpv and 56 dpv after the primary and booster vaccination respectively. At the same time the antibody responses of group II increased continuously after primary and boosted inoculation with purified protein containing empty capsid-like particles. No seroconversion occurred in guinea pigs in group I (Fig. 24).

Table 6

Mean virus neutralizing antibody titres (Log$_{10}$ SN$_{50}$/ 100TCID$_{50}$) against FMDV type 'O' virus, in various groups of immunized guinea pigs at different day post vaccination (dpv)

GROUP	dpv	0	14	28	42 (14 dpb)	56 (28 dpb)
	I (PBS)	0	0	0	0	0
	II (VLPs group)	0	0.91 ± 0.04	1.17 ± 0.12	1.36 ± 0.07	1.40 ± 0.09
	III (FMD-O-Ag)	0	1.08 ± 0.15	1.54 ± 0.23	1.60 ± 0.28	2.08 ± 0.09

The values indicate the mean ± SE

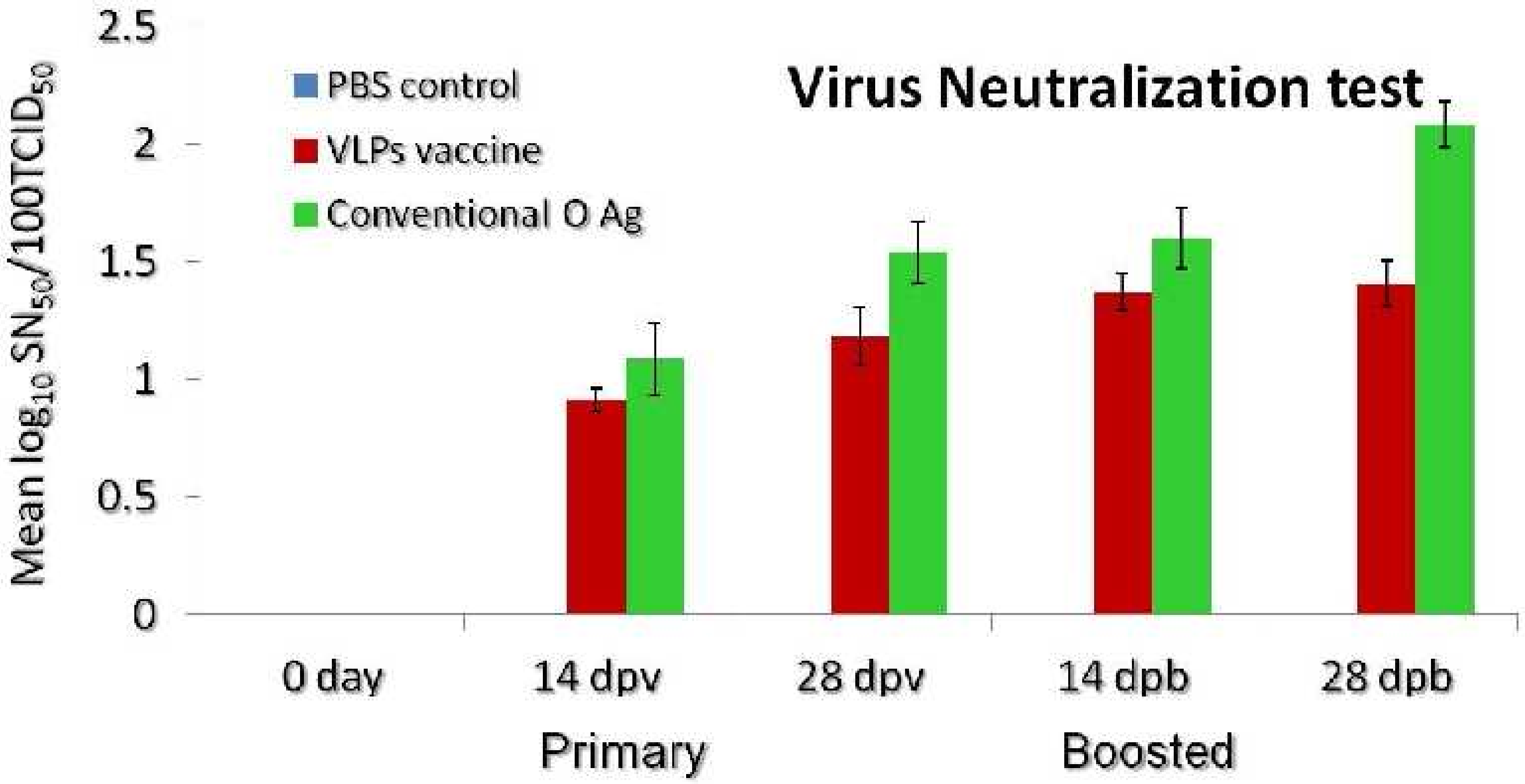


Fig. 23. Mean virus neutralizing antibody titres (Log$_{10}$ SN$_{50}$/ 100 TCID$_{50}$) against FMDV type 'O' virus, in various groups of immunized guinea pigs at different time intervals

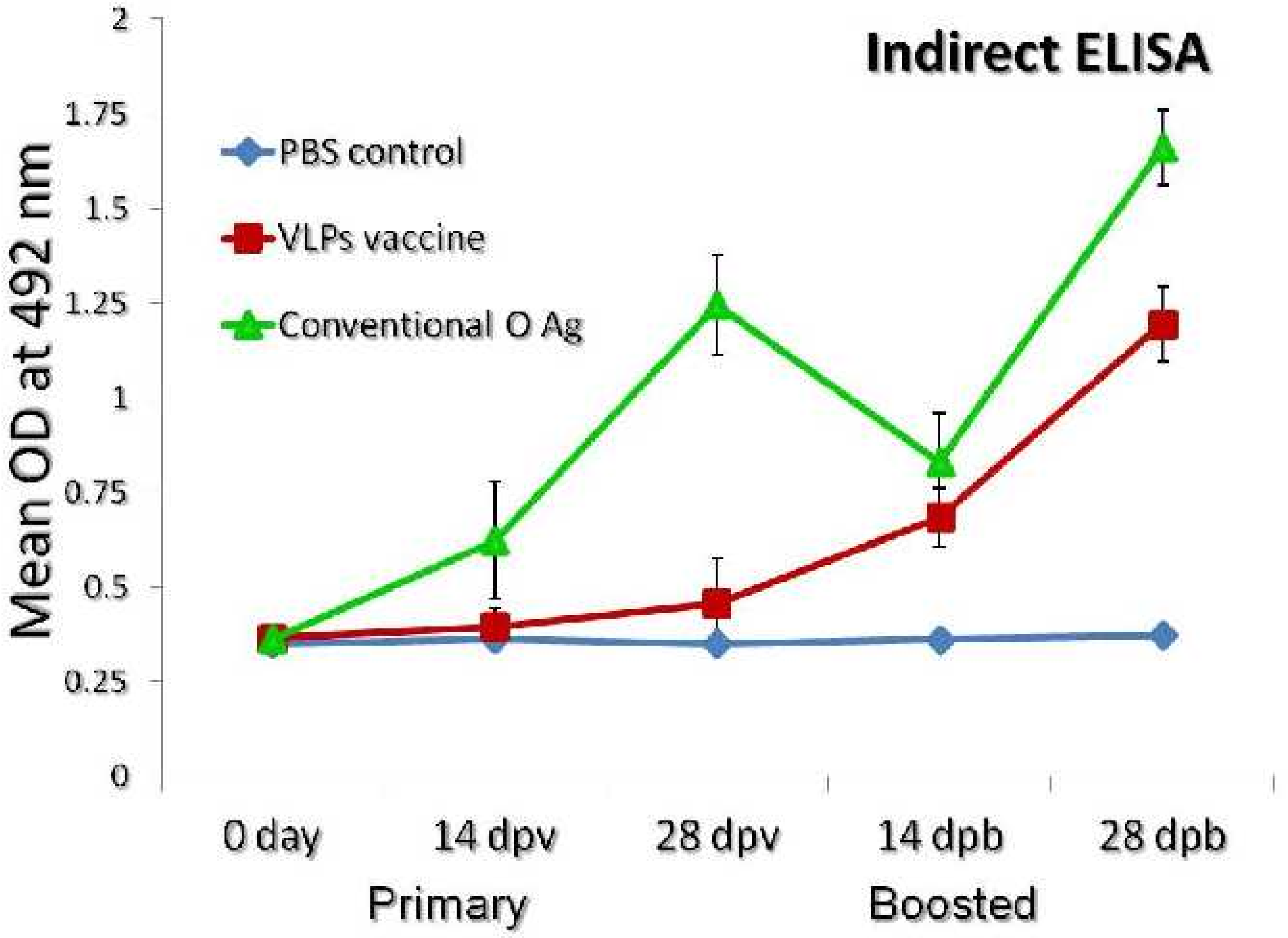


Fig. 24. FMDV type 'O' specific antibody response (Mean OD ± SE) of various immunized groups of guinea pigs at different time intervals in indirect ELISA.

Chapter 5

Discussion

Current FMD vaccine production process involves large scale production and handling of live virus in high bio-containment facilities. In order to avert the risks associated with handling the infectious virus, virus like particles (VLPs) have been considered as alternatives to whole virus particles for use as immunoprohylactics or as diagnostic reagents. Various expression systems such as baculovirus/insect cell, yeast, bacteria are being employed to produce VLPs for a number of infectious agents including FMDV (Lewis *et al*., 1991; Abrams *et al*., 1995; Mayr *et al*., 1999). Production of recombinant VLPs are preferred over whole virus particles as they offer greater advantages. They don’t need expensive bio-containment facility to handle or produce the reagents, and they circumvent the risk of virus escape from production facility. Plus, they facilitate differentiation of infected from vaccinated animals (DIVA). However, some of the limitations in the use of VLPs are lower yield of the antigens in different expression systems tested so far, besides inherently poor immunogenicity of the recombinant antigens when injected in animals. The baculovirus expression system has many advantages over other expression systems, and has been used to produce large quantities of viral proteins (Luckow and Summers, 1989). In FMD empty capsid synthesis, cleavage of precursor polyprotein (P1) of FMDV into structural proteins is mediated mainly by virus coded 3C protease, a non-structural protein. Co-expression of P1 and 3C has been shown to result in processing of P1 precursor capsid into subunits VP0, VP1 and VP3 that self assemble into empty capsids as demonstrated by several groups (Roosien *et al*., 1990; Lewis *et al*., 1991 Porta *et al*., 2013b). Assembled capsids behave antigenically similar to that of FMDV virus particles and therefore can be used as candidate immunogens (Lewis *et al*., 1991) or as reagent to detect FMDV-specific antibodies (Basagoudanavar *et al*., 2013). For efficient expression of empty capsids, a higher level of 3Cpro has proved to have adverse effects on the yields of processed capsids (Polacek *et al*., 2013). This may result from the fact that the FMDV 3Cpro has a number of cellular targets including certain translation initiation factors (eIF4G and eIF4A) (Belsham, 2000), cytoskeleton components (Armer *et al*., 2008) and histone H3 (Falk *et al*., 1990) leading to inhibition of protein synthesis. In our lab, decreased protease activity of 3C as achieved by mutation in 3C has reasonably improved the yield of processed capsids by co-expressing with P1-2A (Basagoudanavar *et al*., 2013).

It has been reported that equimolar amounts of the 3Cpro are not required to achieve efficient processing of the P1-2A, by co-transfecting plasmid containing structural gene and different concentration of 3C plasmid within both insect and mammalian cells (Polacek *et al*., 2013; Porta *et al*., 2013a; Porta *et al*., 2013b). Limiting the expression levels of 3C protease in tandem with P1-2A polypeptide is critical to enhance the yields of VLPs.

In the present study, two approaches were employed both targeted to have reduced levels of 3C relative to the P1 polyprotein in insect cells. One approach was employing IRES intervening P1 polyprotein and 3C cloned in transfer vector (Fig. 2) and another employing dual expression system to mediate expression of two genes under separate promoters. In the first approach, initiation of translation mediated by IRES elements represents an alternative to the cap-dependent translation initiation mechanism used for most cellular mRNAs. Mizuguchi *et al*. (2000) have shown that IRES-dependent second gene expression can be significantly lower than cap-dependent first gene expression in bicistronic vectors. Presence of IRES in the dicistronic mRNA not only drives expression of the downstream reporter gene but also positively influences first transgene expression (Jünemann *et al*., 2007). IRES-dependent translation initiation can bypass stress conditions that are inhibitory for cap-dependent translation initiation (Martínez-Salas *et al*., 2001). Such stress conditions occur following eIF4G cleavage that is induced during picornavirus infection as a consequence of the action of viral proteases Lb (FMDV) or 2A (poliovirus). So in this strategy, two different virus IRES sequences were used for the dicistronic translation; FMDV IRES (3 Indian vaccine strains) and EMCV IRES, the latter widely used to link two genes transcribed from a single promoter within recombinant viral vectors (Martinez-Salas *et al*., 2001).

Preliminary experiment was carried out with bicistronic expression of construct containing P1-2A and a reported gene, GFP intervened with viral IRES sequence derived from either FMDV or EMCV. Expression of GFP could be observed in insect cells infected with the construct carrying FMDV IRES sequence upstream of GFP reporter gene (2nd transgene). FMDV IRES mediated expression of $3Cpro^{wt}$ successfully showed specific cleavage of the capsid precursors (~95 kDa) to processed structural proteins of VP1/VP3 (~28 kDa) and VP0 (~36kDa). It was further evident that, use of $3Cpro^{mut}$ with reduced 3C proteolytic activity (in place of $3Cpro^{wt}$) under FMDV IRES showed reduced cleavage of

P1-2A precursors (i.e) causing increased band intensity of P1-2A polyprotein and VP3+VP1 intermediate precursor protein. This mutant 3Cpro^{mut} (G38S and F48S) gene has been used earlier in this lab (Basagoudanavar *et al*., 2013) to reduce the 3C protease activity to produce the VLPs by expressing P1-2A-3C as a single transcriptional unit. But in FMDV IRES mediated cap independent translation of 3Cpro^{mut}, its activity was further reduced leading to apparent incomplete processing of capsid precursors. Use of GFP gene in place of 3Cpro^{wt}/ 3Cpro^{mut} showed no cleavage of P1-2A precursors in immunoblot assay, as expected. Constructs containing IRES derived from all the three serotypes behaved similarly and showed almost identical efficiency when infected at same MOI in insect cells. By sequence analysis, the percent identity of 92.83%, 91.99%, 89.78% alignment score was observed between O and A, O and Asia1, A and Asia1 IRES, respectively. By RNA secondary structures prediction analysis the structural analogy was conserved at different motifs between these three serotypes of FMDV IRES. Expression kinetics of recombinant capsid protein at different time interval was monitored and it was found that the maximum expression of capsid protein was observed at 72 hours post-infection in Tn5 insect cells.

Translational efficiency of FMDV IRES in mammalian host system was also evaluated. For this adenovirus vector system was used to express the cDNA cassette containing capsid protein precursor (P1-2A) and 3Cpro^{wt}, with type A FMDV-IRES sequence sandwiched between them. Expression of the P1-2A-IRES-3C^{wt} using recombinant adenovirus in HEK 293 cells was assessed by SDS-PAGE followed by western blot. It was observed that 3C expressed under the FMDV IRES sequence was functional with relatively a lower yield of VP1/VP3 protein in comparison to expression of this construct in baculoviral system. Over processing of the capsid components (i.e. VP0, VP1 and VP3) may be ascribed to higher efficiency of FMDV IRES in mammalian cells as compared to insect cell host system. Gullberg *et al*. (2013) have recently shown that it is essential to subdue the activity of IRES in mammalian cells by specific mutation in IRES sequence used in between the two ORFs in such bi-cistronic system.

Similar experiment employing EMCV IRES in a bicistronic expression in baculoviral expression system showed no detectable GFP expression or specific cleavage of the capsid precursors co-expressed with 3Cpro^{wt} under the EMCV IRES. This observation was consistent with previous studies by Woolaway *et al*. (2001), that the picornavirus- derived IRES does not work in insect cells. This was interesting observation despite reasonable degree of structural analogy between FMDV IRES and EMCV IRES as studied by RNA secondary structure prediction analysis, as different motifs between them were conserved along with 48-52% sequence homology observed between FMDV and EMCV IRES elements. Han and Zhang (2006) showed shrimp white spot syndrome virus (WSSV) IRES mediated translation efficiency of about 98% in insect cells. Borman *et al.* (1997) compared the capacities of different picornaviral IRESes to mediate internal translation initiation in a variety of cultured cells. EMCV and FMDV, which were 60–80% efficient in directing internal translation initiation in Hela cells. EMCV element, which was considerably less efficient than the FMDV or HCV IRESes in BHK-21. So by this experiment FMDV IRES was functional in the construct containing P1-2A and GFP or 3Cpro^{wt} or 3Cpro^{mut}. Thus different IRES will have different translational efficiency in depending on the host cell (Borman *et al.*, 1997).

Second approach employed use of relatively a stronger promoter (Ph) to express P1 precurson protein and relatively a weaker promoter P10 to express 3C protease using dual expression system in insect cells as shown earlier (Chung *et al.*, 2010; Cao *et al.*, 2009). In this study, one of the constructs that had P1-2A and 3Cpro^{wt} under separate promoters (Construct 6) failed to give processed capsids based on western blot and ELISA results. This observation was consistent with observation by Ruiz *et al.* (2014) who have proved that infection with a dual-promoter baculovirus vector coding for the capsid precursor (P12A) and the protease 3C under the control of different promoters, respectively allowed lower levels of empty capsid expression in comparison with single-promoter vector coding the P1-2A-3C cassette. Expression of P1-2A and 3C under separate promoters with serotype Asia 1 was initially shown to form empty capsids (Cao *et al.*, 2009). Subsequently, another construct having two copies of P1-2A precursor protein and one copy of 3C was expressed and checked for its yield by western blot. This construct (Construct 7) showed a complete capsid polyprotein processing with higher band intensity of VP1/VP3 than the other constructs.

By VLP quantitative analysis, two constructs gave higher yield based on purification of the VLPs and their antigenicity based on ELISA results. Construct 7 (P12A/P12A-3C) had ~8 fold and ~64 fold better VLP yield than P12A-IRES-3Cpro^{wt} and P1-2A-3C constructs respectively. The results presented here clearly demonstrate, that an enhancement in the yield of processed FMDV capsid proteins could be achieved, by reducing the levels of the 3Cpro relative to the level of the P1-2A substrate. No precise quantification of the 3Cpro generated in these experiments could been performed. Results of 3Cpro^{mut} (in place of 3Cpro^{wt}) (Construct 8) cleavage was comparable and had comparatively less proteolytic cleavage of P1-2A than 3Cpro^{wt} as discussed earlier. However compared to all constructs in this study, higher yield of processed FMDV capsid proteins could be achieved, by increasing availability of the P1-2A as a substrate for the 3Cpro. These purified peak FMDV type O antigenic fractions revealed the authentic typical structure of picornaviral spherical capsids of about size ~25nm diameter under transmission electron microscopy. There was difference in the OD signals detected between the intact precursor and the processed products. Unprocessed P1-2A protein in this study showed higher reactivity in sELISA. This observation is consistent with earlier results, using a P1-2A of FMDV serotype C and serotype O (Sa’iz *et al.*, 1994; Polacek *et al.*, 2013). Thus assembly of FMDV capsid particles is not required for the use of these FMDV antigen detection assays.

In order to study their immunogenicity, guinea pigs were immunized with FMDV empty capsid-like particles as well as with the whole virus inactivated type O FMDV vaccine. Both FMDV-specific antibodies and neutralizing antibodies were generated in the guinea pigs, but their levels were lower than those generated by the commercial vaccine. One possible reason for this was that quantity of empty capsid-like particles might have been lower than in the conventional inactivated vaccine and higher antigenic mass would be required to elicit immune response comparable to whole virus based immunogen. Nevertheless two of the strategies studied here appear to be promising in improving the yields FMD VLPs and this could be potentially exploited in development of improved molecular vaccines and diagnostics for FMD.

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Chapter 4

Summary and Conclusions

FMDV subunit vaccine based on empty capsid-like particles has been developed as one of the most promising alternatives to conventional vaccines (Li *et al*., 2008). These recombinant non-infectious FMDV empty capsid-like particles are potentially useful for the development of diagnostics and vaccines owing to presence of the conformational epitopes as in native antigens, with ability to generate similar immune responses as whole virus antigens (Kushnir *et al.*, 2012). Though studies so far have shown that co-expression of 3C is essential for processing P1 polyprotein into individual subunits and subsequent assembly into empty capsids or virus like particles (VLPs), yields of VLPs is considerably low. Lower yields have been attributed to toxicity and protease activity of 3C which is nevertheless required to process the P1 polyprotein for formation of VLPs. In this study, we have analysed different strategies to determine the optimal system for the production, processing and assembly of FMDV capsid proteins within insect cells by baculoviral expression system. In this experiment we identified the functional IRES sequences from three FMDV serotypes (O, A & Asia 1) to demonstrate cap independent mediated protein expression in insect cells. Study showed that FMDV IRES were functionally recognised in insect cells though to lesser extent than in mammalian cells. However EMCV IRES was not functional in insect cells an observation akin to previous report. Though there was presence of similar sequence motifs, only 48-52% sequence identity was observed between FMD IRES (3 serotypes) and EMCV IRES. The IRES from three Indian FMDV serotypes (O, A and Asia-1) showed similar pattern in 3C mediated P1-2A processing, though they shared 8% sequence divergence between them.. Use of mutant 3Cpro with reduced catalytic activity under FMD IRES motif had sub-optimal proteolytic cleavage of capsid precursors than native 3C. The FMD IRES element in mammalian cells showed apparent over processing of P1 precursor leading to decreased accumulation of capsid sub units (VP0, VP1/VP3) in HEK 293 cells using adenovirus expression system. This underscores that activity of IRES needs to be regulated through mutation in the IRES sequence as shown recently in mammalian system.

Second approach employed use of relatively a stronger promoter (Ph) to express P1 precursor protein and relatively a weaker promoter (P10) to express 3C protease using dual expression system in insect cells as shown by earlier workers. In this study, one of the constructs that had P1-2A and 3Cpro^{wt} under separate promoters (Construct 6) failed to give processed capsids based on western blot and ELISA results. By VLP quantitative analysis, two constructs gave higher yield based on purification of the VLPs and their antigenicity based on ELISA results. Construct 7 (P12A/P12A-3C) had ~8 fold and ~64 fold better VLP yield than P12A-IRES-3Cpro^{wt} and P1-2A-3C constructs respectively. The results presented here clearly demonstrate, that an enhancement in the yield of processed FMDV capsid proteins could be achieved, by reducing the levels of the 3Cpro relative to the level of the P1-2A substrate. No precise quantification of the 3Cpro generated in these experiments could been performed. Results of 3Cpro^{mut} (in place of 3Cpro^{wt}) (construct 8) cleavage was comparable and had comparatively less proteolytic cleavage of P1-2A than 3Cpro^{wt}. However compared to all constructs in this study, higher yield of processed FMDV capsid proteins could be achieved, by increasing availability of the P1-2A as a substrate for the 3Cpro. These purified peak FMDV type O antigenic fractions revealed the authentic typical structure of picornaviral spherical capsids of about size ~25nm diameter under transmission electron microscopy.

In order to study their immunogenicity, guinea pigs were immunized with FMDV empty capsid-like particles as well as with the whole virus inactivated type O FMDV vaccine. Both FMDV-specific antibodies and neutralizing antibodies were generated in the guinea pigs, but their levels were lower than those generated by the commercial vaccine. The animals vaccinated with VLPs exhibited highest mean $\log_{10}$ SN_{50} against FMDV type O with titre of 1.40 ± 0.09 on 56th days post vaccination. Two of the strategies employing IRES sequence and two copies of P1 precursor protein with one copy of 3C here appear to be promising in improving the yields FMD VLPs and this could be potentially exploited in development of improved molecular vaccines and diagnostics for FMD.

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Chapter 7

Mini Abstract

Current FMD vaccines based on inactivated virus antigens have been widely used for control of FMD. Over last few years, research has been ongoing to produce non-infectious, safer vaccine for FMD. Vaccine based on virus like particles (VLPs) has been considered as one of the most promising alternatives to conventional vaccines. Studies have shown that co-expression of 3C is essential for processing P1 polyprotein into individual capsid subunits for assembly of VLPs. However yields of VLPs, in the studies so far, are generally low. This has been attributed to higher proteolytic activity of 3C. Indeed low levels of 3C are sufficient to process P1 polyprotein. Taking this into account, several constructs were made in this study to modulate the expression levels of 3C protease in tandem with P1-2A polypeptide. The objective was to obtain reduced levels of 3C, which is critical to get increased yields of VLPs. Two constructs which showed higher yields of VLPs were selected. These include one containing IRES sequences between P1 polyprotein and 3C (P1-2A-IRES-3C) and the other having two copies P1-2A and one copy of 3C (P1-2A/P1-2A-3C). Inclusion of IRES in the construct (P1-2A-IRES-3C) was expected to have enhanced expression of upstream sequence while the sequence downstream is less expressed. Expression of 3C in this construct was evident as specific cleavage of P1-2A could be observed; highlighting that FMDV IRES was functionally recognized in insect cells. However, as reported previously, a similar construct with EMCV IRES sequence was not functional in insect cells. Both the constructs showed higher yields of VLPs and antigenicity. Formation of empty capsids could be demonstrated by transmission electron microscopy in both the constructs. Purified fractions of VLPs derived from these two constructs showed that one of the constructs (P1-2A/P1-2A-3C) showed higher yields when compared to the construct containing FMDV IRES. On immunization of guinea pigs with the VLPs expressed by both the constructs, neutralizing antibodies were observed, albeit at lower levels when compared to those generated by the conventional FMD vaccine. Lower immunogencitiy of VLPs could be attributed to suboptimal dose of recombinant antigen used for immunization. Additional studies with higher dose of recombinant VLPs need to be done to obtain the desired levels of immune response. In conclusion, the two strategies employed in this study efficiently enhanced the expression of FMDV VLPS in insect cells. This knowledge would be useful in development of improved molecular vaccines and diagnostics for FMD.

Chapter 8

लघुसार

खुरपका एवं मुंहपका रोग (F.M.D) नियंत्रण के लिए, निष्क्रियकृत विषाणु प्रतिजनों पर आधारित, नवीनतम खुरपका एवं मुंहपका रोग (एफ.एम.डी.) टीकों को ही आजकल विस्तृत रूप से उपयोग में लाया जा रहा है। तथापि, नॉन-संक्रामक एवं सक्षम टीका उत्पादन के लिए, विगत कतिपय वर्षों से किए जा रहे अनुसंधान-प्रयास आज भी सतत रूप से जारी हैं। परम्परागत टीकों की तुलना में, विषाणु जैसे कणों (VLPs) पर आधारित टीका, अत्यंत प्रभावकारी विकल्पों में से, एक सक्षम विकल्प समझा जाता रहा है। किए गए अनेकों अध्ययनों से यह विदित होता है कि P1 पॉलि-प्रोटीन के विदलन के लिए, कैप्सीड उपएककों (सबयूनिट्स) में एवं VLPs की अगली असेम्बली में, 3C की सह-अभिव्यक्ति अत्यंत अनिवार्य है। तथापि, अब तक किए गए समस्त अध्ययनों में, VLPs की उपज, सामान्यतया न्यून-स्तरीय ही पायी गई है , जोकि प्रोटीन-अपघटक गतिविधि को उच्चतर स्तर की बनाता है। वास्तव में, P1 पॉलि-प्रोटीन के क्रियान्वयन के लिए, 3C के न्यून-स्तर ही पर्याप्त हैं । इस तथ्य को ध्यान में रखते हुए, P1-2A पॉलिपेप्टाइड के अनुबद्ध के साथ में, 3C प्रोटीज के स्तरों की अभिव्यक्ति को मोडूलेट करने के लिए प्रस्तुत अध्ययन में अनेकों संघटक तैयार किए गए। इन संघटकों को तैयार करने का उद्देश्य यह था कि 3C के स्तरों में कमी लाए जाए, क्योंकि 3C के स्तरों में यह कमी ही VLPs की उपजों को बढाने में क्रांतिकारी कारण रही है। अतैव, प्रस्तुत अध्ययन में, VLPs की उच्चतर उपजों को दर्शाने वाले दो संघटकों का चयन किया गया। पहले वाले संघटक में P1 पॉलिप्रोटीन एवं 3C (P1-2A-IRES-3C) के बीच में IRES श्रेणियां और दूसरे वाले संघटक में P1-2A की दो प्रतिलिपियां और 3C (P1-2A/P1-2A-3C) की एक प्रतिलिपि निहित पायी गईं । (P1-2A-IRES-3C) संघटक में IRES निहित पाया गया, जोकि अप-स्ट्रीम श्रेणियों की उच्चतर अभिव्यक्ति और डाउन-स्ट्रीम श्रेणियों की न्यूनतर अभिव्यक्ति को दर्शाता है। इस संघटक में 3C की अभिव्यक्ति में P1-2A का विशिष्ट विदलन पाया गया, जोकि कीटाणु कोशिकाओं में FMDV IRES के क्रियान्वयन की पहचान करता है। तथापि, पिछले अध्ययनों में, कीटाणु कोशिकाओं में EMCV IRES श्रेणियों के साथ एक संगत संघटक क्रियांवित नही था। इन दोनो संघटकों में, VLPs की उच्चतर उपज के साथ-साथ, अपेक्षित प्रतिरक्षाघनत्व पाए गए। इन दोनो संघटकों में ट्रांमिशन इलेक्ट्रोन माइक्रोस्कोपी द्वारा खाली कैप्सीड का संगठन किया गया। इन दोनो संघटको से VLPs के शुद्धिकृत भिन्नों का व्युत्पन्न यह दर्शाता है कि FMDV IRES संघटक की तुलना में (P1-2A/P1-2A-3C) संघटक में VLPs की उच्चतर स्तर की उपज पायी गई। परम्परागत खुरपका एवं मुंहपका रोग (F.M.D) टीकों से उत्पादित प्रतिरक्षियों की तुलना में, इन दोनो संघटको के द्वारा अभिव्यक्त किए गए VLPs के साथ गिनिया-पिग्स का प्रतिरक्षाकरण करने पर, प्रतिरक्षियों को उदासीन पाया गया। जोकि प्रतिरक्षाकरण के लिए, VLPs का न्यूनस्तरीय प्रतिरक्षा-घनत्व, पुनर्योजक प्रतिरक्षी की उप-इष्टतम खुराक (मात्रा) का महत्व प्रदर्शित करता है। पुनर्योजक VLPs की उच्चतर खुराक के साथ किए गए अनेकों अध्ययनों में यह पाया गया कि प्रतिरक्षा-प्रतिक्रियाओं के अपेक्षित इष्टतम स्तरों की प्राप्ति की, अभी और भी आवश्यकता है। निष्कर्षत:, प्रस्तुत अध्ययन में दो व्यूहरचनाओं को उपयोग में लाया गया है : पहली, कीटाणु कोशिकाओं में FMDV VLPs की सक्षम अभिव्यक्ति और दूसरी, FMDV VLPs की उपज में अभिवृद्धि। परिणामत:, संशोधित आण्विक टीकों के विकास में और FMD के डायग्नोस्टिक्स में, यह जानकारी आगे भविष्य में भी उपयोगी सिद्ध होगी।

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Chapter 9

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APPENDIX

A. BACTERIAL GROWTH MEDIA

LURIA BERTANI (LB) BROTH (pH 7.0)

- 2.5 g of LB dehydrated powder was dissolved in 100 ml distilled water and autoclaved at 121˚C for 15 minutes at 15lbs. For LB agar plates, 1.5% of agar was added to the LB broth and autoclaved at 121˚C for 15 minutes at 15 lbs.

B. REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

TBE BUFFER (10X) pH 8.0

Tris base	108g
Boric acid	55g
EDTA (0.5M, pH 8.0)	40ml

- Distilled water to make the volume to 1000ml

ETHIDIUM BROMIDE

10mg	
Distilled water	1ml

GEL LOADING DYE (6X)

Bromophenol blue	1%
Sucrose in water	40%

C. SOLUTIONS AND BUFFERS FOR SDS-PAGE

SDS-PAGE SAMPLE BUFFER

Tris HCl (0.5%), pH, 6.8	0.5ml
Glycerol	0.4ml
SDS (10%)	0.8ml
β -Mercapto ethanol	0.2ml
Bromophenol blue, 1% (w/v)	0.2ml

- Distilled water to make up the volume to 4ml

SEPARATING GEL (12%)

30% Polyacrylamide	3.6ml
Tris HCl (1.5M), pH 8.8	2.25ml
SDS (10%)	90µl
Ammonium persulphate (10%)	120µl
TEMED	10µl
Distilled water	3.0ml

STACKING GEL (5%)

30% Polyacrylamide	510µl
Tris HCl (0.5M), pH 6.8	375µl
SDS (10%)	30µl
Ammonium persulfate (10%)	30µl
TEMED	3µl
Distilled water	2.04ml

SDS-PAGE RUNNING BUFFER, pH 8.3

Tris base(25mM)	3.0g
Glycine(250mM)	14.4g
SDS (0.1%)	1.0g

- Adjust the volume to 1000ml with distilled water

PROTEIN STAINING SOLUTION

Coomassie Brilliant Blue R-250	0.250mg
Methanol	45 ml
Glacial acetic acid	10 ml
Distilled Water	45 ml

PROTEIN DE-STAINING SOLUTION

Methanol	45 ml
Glacial acetic acid	10 ml
Distilled Water	45 ml

D. SOLUTIONS AND BUFFERS FOR WESTERN BLOTTING

ELECTRODE BUFFER, pH 8.3

Tris base (0.025M)	3g
Glycine (0.192M)	14.4g
SDS(0.1%)	1.0g
Methanol (20%)	200ml

- Make up the volume up to 1000ml with distilled water

TRANSFER BUFFER

Tris HCl, pH 8.3	3g
Glycine	1.44g
Methanol	20ml

- Make up the volume to 100ml with distilled water

PBS-T

PBS (1x), pH 7.6	1000ml
Tween 20	0.5ml

BLOCKING SOLUTION

PBS-T	100ml
Defatted skim milk powder	5.0g

E. MEDIA AND BUFFERS FOR CELL CULTURE AND VIRUS PREPARATION

MODIFIED EAGLE’S MEDIUM (GLASGOW MODIFICATION)

Ingredients required for preparing 1000 ml of the medium.

NaCl	6.40 g
KCl	0.40 g
$CaCl_2.2H_2O$	0.26 g
$MgSO_4 .7H_2O$	0.20 g
$NaH_2PO_4.2H_2O$	0.14 g
Dextrose	4.50 g
Ferric Nitrate	0.0001 g
L-Glutamine	0.292 g
Penicillin	10, 000 I.U

Streptomycin	0.10 g
Phenol Red 1%	1.50 g
Distilled water	500 ml
$NaHCO_3$	2.75 g
Amino acid stock solution	50.00 g
Vitamin stock solution	4.00 ml
Distilled water added up to	1000 ml

- The ingredients were dissolved in order and CO_2 was passed through the solution to adjust pH in between 7.2 to 7.4. The medium was sterilized by filtrating through Seitz-EKS pads and stored at 4°C till use.

STOCK SOLUTION OF AMINO ACIDS (1000 ml)

L. Arginine	0.84 g
L. Cystine	0.48 g
L. Histidine	0.38 g
L. Isoleucine	1g
L. Leucine	1.048 g
L. Lysine	1.462 g
L. Phenylalanine	1.600 g
L. Threonine	0.952 g
L. Tryptophan	0.160 g
L. Valine	0.936 g
Phenol red 1%	0.04 ml
L. Tyrosine	0.936 g

- Dissolved separately in 25 ml of N/1 NaOH solution. The ingredients were dissolved by heating to 56°C and distributed in 250 ml screw capped bottles and stored at –20°C.

STOCK SOLUTION OF VITAMINS (1000ML)

Choline chloride	500 mg
Folic acid	500 mg
Nicotinamide	500 mg
Pantothenic acid	500 mg
Riboflavin	500 mg

Inisitol	350 mg
Pyridoxal HCl	500 mg

- The ingredients were dissolved properly and distributed and stored at –20°C.

RPMI-1640 MEDIUM

(With L-Glutamine, without Glucose and sodium bicarbonate, product number R1383)

Preparation as per the instructions given by the manufacturer.

TRYPTOSE PHOSPHATE BROTH (TPB), pH 7.3

Tryptose	20 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g

- The ingredients were dissolved in 1000ml distilled water and boiled to dissolve the them completely. TBP was sterilized by autoclaving at 121˚C at 15 lbs for 15 minutes.

TRYPSIN VERSENE GLUCOSE (TVG)

Trypsin (Difco; 1:250)	2.0 g

Distilled water to make up to 100 ml

- The trypsin was dissolved by agitating the solution at 4°C for 15 minutes and then filtered through Seitz-KES pads and stored at 4°C.

Versene (AR) /EDTA	0.2 g

Distilled water to make up to 100 ml

- The solution was sterilized by autoclaving at 121°C under 10 lbs pressure for 10 min and stored at 4°C.

Glucose (AR)	10 g

Distilled water to make up to100 ml

- The solution was sterilized by autoclaving at 121°C under 10 lbs pressure for 10 min and stored at 4°C.

Trypsin Versene Glucose (TVG)

PBS	840 ml
Versene (0.2%)	100 ml
Trypsin (2%)	50 ml
Glucose (10%)	5 ml

Phenol red (1%)	1 ml

- The above ingredients were mixed aseptically in a laminar flow and checked for sterility test and stored at 4°C until use.

NORMAL SALINE SOLUTION (NSS)

Sodium chloride	8.5 g
Distilled water	1000 ml

PHOSPHATE BUFFERED SALINE (1X) (PBS) (0.15 M, pH 7.2)

Sodium chloride	8.0 g
$Na_2HPO_4.2H_2O$	1.44 g
Potassium chloride	0.2 g
KH_2PO_4	0.2 g

- Distilled water to make up to100 ml

TRYPAN BLUE SOLUTION (0.1%)

Trypan Blue	0.1 g
Phosphate buffered saline, pH 7.2	100 ml

- Filtered and stored at 4°C

GROWTH MEDIUM

EMEM	900ml
TPB	100ml
Fetal Calf Serum (5%)	50ml

MAINTENANCE MEDIUM

EMEM	950ml
TPB	50ml
Fetal Calf Serum	20ml

F. CESIUM CHLORIDE SOLUTIONS FOR ULTRACENTRIFUGATION

1.38g/ml DENSITY SOLUTION

$CsCl_2$	5.095g

Tris NaCl, pH 7.6 made up to 10 ml

1.42g/ml DENSITY SOLUTION

$CsCl_2$	5.678g

Tris NaCl, pH 7.6 made up to 10.0 ml

Tris – NaCl BUFFER, pH 7.6

Trizma HCl	1.212 g
Trizma Base	0.278
NaCl	4.384 g

- Distilled water up to 500 ml

G. REAGENTS FOR ELISA

COATING BUFFER

***Solution A*:** 0.2 M Sodium carbonate solution

Na_2CO_3	21.198 g

- Distilled water to make up to 1000 ml

***Solution B*:** 0.2 M Sodium bicarbonate solution

$NaHCO_3$	16.8 g

- Distilled water to make up to 1000 ml

Working Solution: 16 ml of solution A and 32 ml of solution B were mixed and the volume was made up to 200 ml with triple distilled water and stored at 4°C.

WASHING BUFFER (PBS-TWEEN 20), pH 7.2

1X PBS	1000 ml
Tween 20	0.5ml

BLOCKING BUFFER

PBS (0.1 M), pH 7.4	100ml
Tween 20	0.05ml
Skim Milk Powder (SMP)	5g

SUBSTRATE SOLUTION

Citrate buffer, pH 5.0

Citric acid	7.30 g
$Na_2HPO_4. {}_2H_2O$	11.87 g

- The volume was made up to 1000 ml with distilled water and stored at 4°C

WORKING SOLUTION (pH 5.0)

Citrate buffer	6 ml
OPD	4 mg
30% H_2O_2	8 µl

STOP SOLUTION (1M H_2SO_4**)**

Conc. H_2SO_4	5.56 ml

- Volume was made up to 1000ml with distilled water and stored at room temperature

OTHER SOLUTIONS

TE BUFFER (1X)

Tris HCl, pH 8.0	10mM
EDTA, pH 8.0	1mM

- Molecular grade water was used to prepare the solution and sterilized by autoclaving at 121ºC for 20 minutes at 15 lbs on liquid cycle and stored at 4˚C

GLYCEROL PHOSPHATE BUFFER, pH 7.6

Sterile Glycerol	50 ml
Sterile 1X PBS	50 ml

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Curriculum Vitae

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