NOVEL DIAGNOSTIC APPROACHES FOR DETECTION OF KYASANUR FOREST DISEASE VIRUS

A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree

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SUCHI AGRAWAL

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **NOVEL DIAGNOSTIC APPROACHES FOR DETECTION OF KYASANUR FOREST DISEASE VIRUS** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2018 to June 2019 under the supervision of Dr. Debasis Nayak, Assistant Professor.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

(SUCHI AGRAWAL)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

(DR. DEBASIS NAYAK)

SUCHI AGRAWAL has successfully given her M.Sc. Oral Examination held on **June 26, 2019.**

Signature of Supervisor of MSc thesis
Date:

Convener, DPGC
Date:

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Date:

Signature of PSPC Member #2
Date:

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"Research is to see what everybody else has seen, and to think what nobody else has thought"

- ***Albert Szent-Gyorgyi***

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Suchi Agrawal

DEDICATED TO

MY FAMILY

Abstract

In 1957, an epidemic broke off in the Kyasanur forest area of Karnataka state killing numerous primates. Today, this febrile illness is responsible for more than 400-500 human cases per year in India. Kyasanur Forest Disease (KFD) is one of the most understudied tick-borne disease. Recently, there has been upsurge in the number of cases with more frequent outbreaks and reports of disease coming from previous unreported areas. This should serve as a reminder about number of health concerns it poses with no therapeutic options available for combating the disease. Research on Kyasanur forest disease virus (KFDV), a flavivirus, has also been limited due to BSL 4 categorization. Accurate diagnosis of KFD still largely remains a challenge since it is often misdiagnosed as some related illnesses such as influenza, typhoid, dengue, malaria and rickettsial group of fever. A differential diagnostic tool which is sensitive, rapid and robust is critically required. In this study, we aim to target viral antigenic sites to derive pAb. KFDV EDIII domain of viral envelope protein (E) is known to carry epitopic regions for the generation of neutralizing antibodies. Other domains also induce immune response but are known to present cross-reacting antibodies. We show that KFDV EDIII is an immunodominant domain and thus can be effectively used for development of antibody-based differential diagnostic and therapeutic tool for early detection of KFD. In this project, we report that the first bleed anti-KFDV EDIII antibodies showed cross-reactivity whereas terminal bleed anti-KFDV EDIII antibodies was found to be antigen-specific and does not show cross-reactivity with DENV (1-4) and ZIKV viral antigens.

Keywords: Kyasanur forest disease virus, KFDV, envelope protein, flavivirus, Domain III, antibody-mediated neutralization

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ACRONYMS

AHFV	Alkhurma hemorrhagic fever virus
APS	Ammonium persulphate
BSA	Bovine serum albumin
BSL	Biosafety level
CDC	Centre for Disease Control
CDS	Complementarity determining sequence
DENV	Dengue virus
DNA	Deoxy-ribonucleic acid
DTT	Dithiothreitol
E Protein	Envelope protein
EDI	Envelope protein domain I
EDII	Envelope protein domain II
EDIII	Envelope protein domain III
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FP/FL	Fusion peptide/fusion loop
HRP	Horse-radish peroxidase

IFN	Interferon
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JEV	Japanese encephalitis virus
KFD	Kyasanur Forest disease
KFDV	Kyasanur Forest disease virus
LFA	Lateral flow assay
LIV	Louping ill virus
MBFV	Mosquito-borne flaviviruses
MSA	Multiple sequence alignment
NCBI	National Centre for Biotechnology Information
NCR	Non-coding region
NHP	Non-human primate
NIV	National Institute of Virology
NJ	Nanjiyan virus
NKVFV	No known vector flavivirus
NMR	Nuclear magnetic resonance
NVBDCP	National Vector Borne Disease Control Programme
OHFV	Omsk hemorrhagic fever virus
ORF	Open reading frame
PCR	Polymerase chain reaction

PDB	Protein databank
PMSF	Phenylmethylsulfonyl fluoride
POC	Point-of-care
RMSD	Root-mean square deviation
RNA	Ribo-nucleic acid
RSSE	Russian summer-spring encephalitis
RT-PCR	Real-time polymerase chain reaction
SLEV	St. Louis encephalitis virus
TBE	Tick- borne encephalitis
TBEV	Tick-borne encephalitis virus
TBFV	Tick-borne flaviviruses
TEV	Tobacco etch virus
TRX	Thioredoxin
UTR	Untranslated region
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

Chapter 1

Introduction

1.1 Genus *Flavivirus*

The genus *Flavivirus*, together with *pestivirus, hepacivirus* and *pegivirus,* belongs to the family of *Flaviviridae*. It is the largest genus of the *Flaviviridae* comprising about >70 virus species in the group [1]. They are more commonly known as arboviruses due to their mode of transmission through vectors which are generally either mosquitoes or ticks [2]. Known to cause encephalitis, hemorrhagic fevers, flaccid paralysis, biphasic fever and other manifestations in a wide range of host species [3], the members of the genus flavivirus are some of the most notorious human and animal pathogens such as dengue virus (DENV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), Zika virus (ZIKV) and West Nile virus (WNV) etc.

The flavivirus outbreaks are being reported around the world. For example, ZIKV outbreaks have been reported by more than 86 countries and territories since its emergence in 2015 [4]. Similarly, in 2016, Angola, Uganda and Democratic Republic of Congo reported serious outbreaks of yellow fever [5]. Severe to mild cases of dengue fever [6] affecting more than 50 million cases are reported annually [7]. Another member, the JEV is reported to infect up to 50,000 individual annually which cause major inflammatory encephalitis [8]. An unexpected outbreak of a mosquito-borne illness in New York, USA in August 1999 brought into light the West Nile virus as a major continental pathogen [9]. Together, emergence and reemergence of flaviviruses are reported periodically across the globe.

Most of the flaviviral infections are contagious; transmission can be classified based on their natural vector [10]. They can be either mosquito-borne flaviviruses (MBFV), tick-borne flaviviruses (TBFV) or flaviviruses with no known vectors (NKVFV).

 TBFV	 MBFV	Flavivirus with no known vectors (NKVFV)
• Tick-borne encephalitis virus • Louping ill virus • Langat virus • Omsk Hemorrhagic virus • Kyasanur Forest Disease Virus • Powassan virus • Royal Farm virus • Gadgets gully virus • Meaban virus	• Dengue virus serotypes • West Nile virus • Zika virus • Japanese encephalitis virus • Yellow fever virus • St Louis encephalitis virus • Tembusu virus	• Cowbone ridge virus • Entebbe bat virus • Yokose virus • Modoc virus • Carey Island virus

Fig. 1.1 General classification of flaviviruses based on the transmitting vector. These viruses have global distribution and cause significant public health impact and veterinary concerns. As reported, 50% of flavivirus are mosquito-borne, 28% are tick-borne while rest keep circulating among rodents or bats with no known specific vectors and about more than 50% are implicated in human diseases [11].

Majority of tick-borne flaviviral infections generally remain in the forestlands and moorlands, away from human contact. But recent phenomenon of urbanization and deforestation has broken this trend. As reported, every year, between 3,500 and 10,000 cases of tick-borne encephalitis occur in urban and countryside of Russia, chiefly because the city boundaries frequently overlapped with that of forests and other susceptible wastelands [12].

In India, Japanese encephalitis is a major public health problem in the state of Uttar Pradesh, Bihar, Odisha, and North-East states. Together,

Asian sub-continent accounts for more than 16,000 reported cases of Japanese encephalitis of which 5,000 are fatal [13]. Though only four confirmed cases of Zika virus have been recorded in India [4], but the current globalization and travel could worsen the scenario quickly. The National Vector Borne Disease Control Programme (NVBDCP) reported the number of dengue cases in India to be about 74,454 in 2013. And the trend remain similar in following years.

Because of this reason, the international community has called for accelerating the research on these type of diseases. Though many of these viruses have been known since long, as early as 1900s, but for the first time outbreaks of this magnitude have been observed recently. These current trends of spread of vector-borne flaviviral epidemics have rose due to much greater vector density, especially in urban settings.

1.2 Kyasanur Forest Disease Virus (KFDV)

KFDV belongs to tick-borne encephalitis sero-complex of flaviviruses. It is a major zoonotic flavivirus responsible for viral hemorrhagic fever endemic to Karnataka state of India. The KFDV was first recognized and isolated from the Kyasanur forest in the Shimoga district of Karnataka in 1957 during outbreak of the febrile disease among neighboring people [6,10] and hence derived its name. Pioneer serological studies suggested that the novel fever was caused by a virus closely related to Russian summer-spring encephalitis (RSSE) complex of arboviruses, now known as tick-borne encephalitis (TBE) complex of flaviviruses [16].

1.2.1 Geographical distribution of KFD virus

Initially, KFD was confined to 3 taluks of the Shimoga district of Karnataka until 1972. Recent reports suggest the presence among other unreported parts of India including Saurashtra region of Gujarat, parts of Kerala and Tamil Nadu, Goa, forested regions west of Kolkata and

Andaman Islands [14]. This may be either due to the availability of better diagnostic tools [17] and improved surveillance or may manifest as altered virus transmission due to climatic changes and disturbances and change in eco-biology of the virus [14]. Variants of KFDV have also been found in Saudi Arabia, named as Alkhurma hemorrhagic fever virus (AHFV) and Chinese province of Yunan, named Nanjiyan virus (NJ).

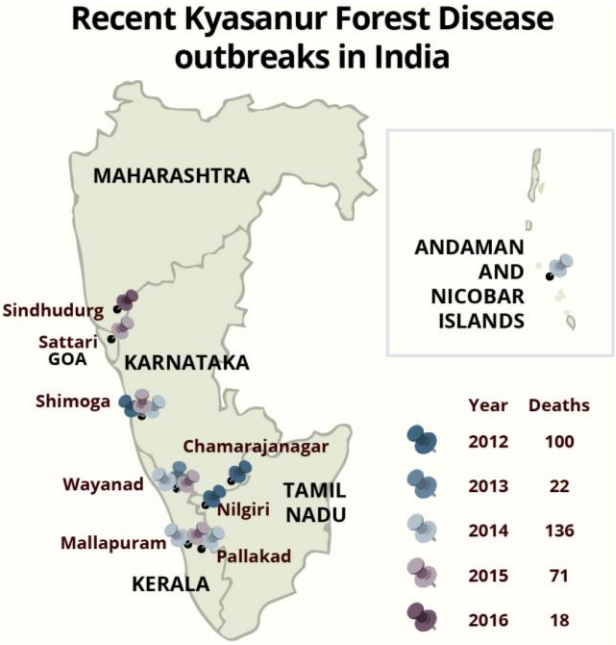


Fig. 1.2 Geographical distribution of recent Kyasanur Forest disease outbreaks in India as of 2016. An empirical map showing the sporadic spread of KFD across Southern states; mainly along Western Ghats during the time period between 2012-2016 (adapted from Mourya *et al.*, 2016) [20].

Since 1972, the incidence of KFD in India has been around 400-500 cases per year [14] with the mortality rate of 5-10% [15]. Between 2003 and March 2012, about 3,263 human infection of KFDV are reported [14]. The epidemic, starting from November or December, peaks from January to April and declines by June and July.

Recently, Government of Karnataka has issued high alert over the outbreak of KFD which is going on since December, 2018 with sporadic cases reported across the state. According to a *Times of India* report published on June 17, 2019, the Shimoga district reported a total of 3,548 KFD cases in 2018-19 of which 341 were found sero-positive. Also during this time, 491 monkeys died and of these 21 sero-positive cases were reported.

1.2.2 Mode of transmission of KFD virus

Immunologically close to Alkhurma virus, KFDV is a zoonotic pathogen transmitted by the tick vector species *Haemophysalis* which serves both as vector and reservoir for the infection. Small vertebrates such as rodents are also the reservoir of the virus. *Haemophysalis spinigera* is isolated from about >95% of cases primate infection in the Karnataka [21]. Humans play the role of secondary host. KFDV is known to induce both cell-mediated and humoral immune responses inside the host. In natural infection cycle, ticks transmit the viruses to a non-human primate (NHPs) [14]. These NHPs, especially black-faced langurs [20] and red-faced bonnet macaques [22] are highly susceptible and usually succumb to KFDV infection. Till date, human-to-human transmission is not known although persons directly handling sick monkeys or lab personnel or villagers living close to affected forest areas become dead- end host to viral infection [14].

1.2.3 Clinical signs and symptoms of KFD

Clinically, the viral infection manifests three conditions: encephalitis, hemorrhagic fever and acute febrile illness. Incubation period for KFD is about 2-7 days after tick bites or exposure. Sudden onset of illness is characterized by headache, fever, chills and myalgia [23]. Hemorrhages in the gastro-intestinal tract and oro-nasal cavity are soon followed.

1.2.4 Disease diagnosis

Because of close clinical symptoms to other infectious disease, confirmatory diagnosis should be differentiated from related illnesses such as influenza, typhoid, dengue, malaria, leptospirosis and rickettsial group of fever. Viral genome detection is currently accepted gold standard technique for confirmatory diagnosis of KFD. For this, Real-time PCR-based assays are developed for detection of viral nucleic acids from human as well as monkey blood samples. Other than this, serological techniques such as detection of anti-KFDV IgM antibodies (ELISA-based) is employed to detect viral presence during the acute phase of illness [14]. During KFDV infection, the level of viremia reaches up to 3×10^6 just within 3–6 days during the acute phase of illness and remains high for the period of 10–14 days of infection [22]. As IgM is not virus specific, it could result in false positive/negative interpretation. Similarly, detection of genomic RNA through multi step procedure involving RNA isolation, cDNA synthesis, real-time PCR is cumbersome process requiring standard operating protocols. This demands skilled man-power and infrastructure. Hence there is a need for alternate technology which would effectively provide early diagnostic assessment with higher sensitiveness and robustness. Since viral presence is reported during the 3^{rd} to 14^{th} days in infected individual, direct detection of viral antigen could be an attractive technique for KFDV confirmation.

1.2.5 Treatment available

According to the U.S. Centre for Disease Control (CDC), KFDV is a BSL-4 pathogen. Due to large number of laboratory-associated infections related to KFD at NIV, Pune, work on the virus was halted for over 30 years and resumed when BSL-4 facility was established [14]. No treatment is available for KFD; only supportive therapy, including maintenance of hydration, hemodynamic stability and coping with neurological symptoms can help manage the infection. Studies have

shown that KFDV has the capability to overcome the host antiviral response such as that of IFN [23].

1.2.6 Disease prevention

Adequate preventive measures are lacking to control KFD spread. Formalin-inactivated virus grown in chick embryo fibroblast tissue culture developed in 1990 is currently available as a candidate vaccine. This formulation is currently used to prevent KFD in endemic areas [24] and being manufactured under the aegis of Government of Karnataka, India. Certain disadvantages, such as inherent risk of utilizing pathogenic viruses and potential chances of incomplete attenuation of the viral particle, linked with the live attenuated vaccines. Moreover, the vaccine regimen that is followed includes booster doses at interval of 6-9 months after first series of vaccination and subsequent boosting every 5 years giving rise to patient compliance issues [16]. Taken together, these observations necessitates the development of new and more effective vaccination strategy.

Chapter 2

Review of Literature and Problem Formulation

2.1 *Flavivirus* genome organization and role of envelope glycoprotein

KFDV belongs to the group of single stranded positive-sensed RNA viruses having genome size of ~10.4 kb (NCBI accession no: NC_039218). Flaviviruses have an average virion size of diameter of 50nm [25]. The genome comprises of a single open reading frame (ORF) encoding a polyprotein and flanked on both sides by 5'and 3' untranslated regions (UTRs) [26]. The 3' UTR is devoid of poly-(A) tail and the 5' UTR contains type I cap structure (m^7 GpppAm) in which guanine at N-7 position and 2'-OH of adenine (first nucleotide of mRNA) are methylated [27]. The polyprotein is cleaved by different host cellular and viral proteases to yield functional structural and non-structural proteins. The structural proteins, on cleavage with proteases give rise to three proteins:

1) C: a nucleocapsid protein
2) prM: a precursor membrane (pre-membrane) glycoprotein
3) E: a glycosylated envelope protein

Likewise there are 7 non-structural protein are also encoded, namely: NS1, NS2A/B, NS3, NS4A, 2K, NS4B and NS5. The nonstructural protein 5 (NS5) mediates the methylations of genome at 5' UTR [28].

The nucleocapsid protein C is responsible for coating of genetic material and forms the viral capsid. The pre-membrane glycoprotein is responsible for maintaining the conformational structure of the envelope (E) protein

and plays an important role in shaping the viral envelope [29]. The envelope protein along with the pre-membrane protein shapes the surface of virion [30]. The main viral structural protein is envelope protein E which facilitates membrane fusion between the virus and host cell [31]. This protein is highly immunogenic in nature. It is one of the major protein against which neutralizing antibodies are generated in the infected host [32]. The non-structural proteins coordinate the cellular processes as maturation, assembly, replication, proteolysis and antagonize host immune response [29].

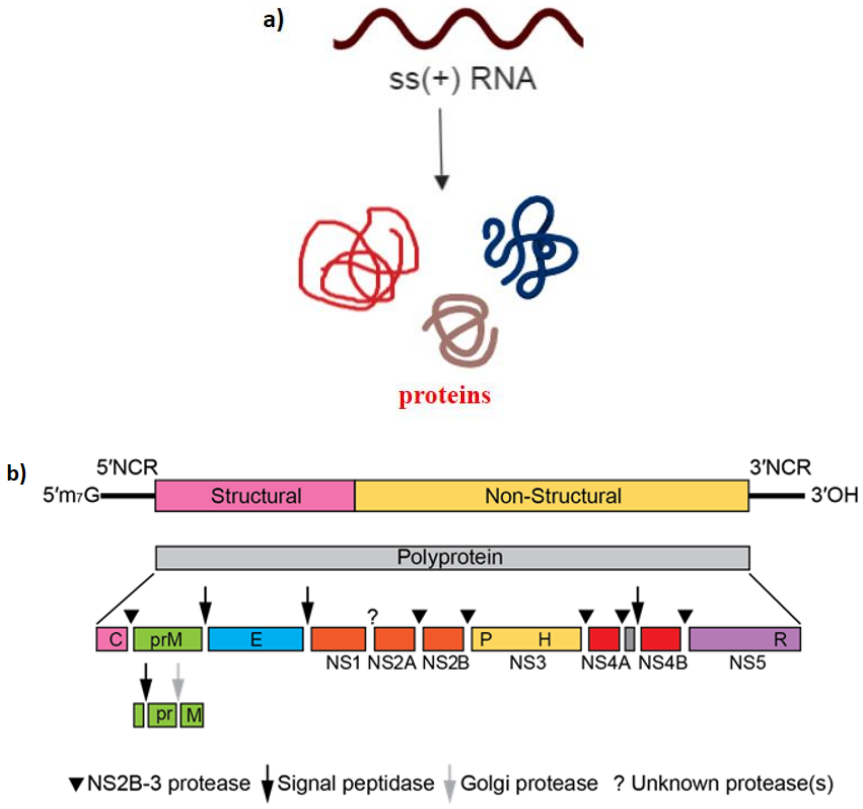
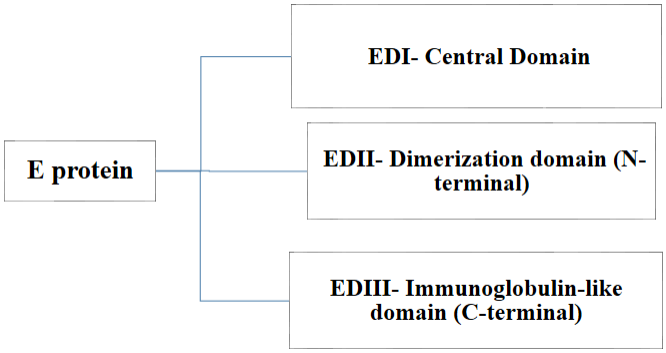


Fig. 2.1 ***Flavivirus*** **genome structure.** a) According to Baltimore virus classification system, flaviviruses are classified under Group IV viruses with (+)ssRNA as genome [33]. b) The ORF encoded polyprotein is flanked on both sides with non-coding regions (NCRs) or UTRs and is processed into three structural and seven non-structural proteins by a range of host and viral proteases during the process of virion maturation (adapted from *ICTV)* [11].

E protein is structurally identified by using combination of electron cryo-microscopy [34], X-ray crystallography [35] and NMR spectroscopy [36], The E protein monomers have been shown to be divided into three distinct domains; domain I, II and III.



Exposure to low pH in the host endosomes leads to trimerization of the E protein and this exposes a highly conserved hydrophobic and glycine rich fusion peptide (FP) or fusion loop [37] at the tip of domain II (EDII) [38]. The E protein is a typical class II viral membrane fusion protein, rich with β-sheets. Typically this forms a pre-fusion homodimer that inserts highly conserved fusion loops into the host membranes resulting in post fusion trimers [31].

A single glycosylation site is present in flaviviruses E protein at Asn-154 position while in DENV two glycosylation sites are present [39]. The three conformational non-linear domains (I, II, III) of the glycosylated E protein are involved in different functions. Domain I or central domain stabilizes overall orientation of the E protein. It also participates in conformational changes of the protein in a pH-dependent manner. The conserved glycosylation of E protein is important for virus production in the host and believed to be associated with neuro-invasion in the infected host [40]. Domain II or dimerization domain has fusion loop at its N-terminal which is highly hydrophobic and is involved in virus-entry. It plays a major role in virus-mediated membrane fusion and thus important in virus infection initiation process. Domain II contains cross-reactive peptides and also are

known to stimulate production of neutralizing antibodies [1] in the immune-competent host.

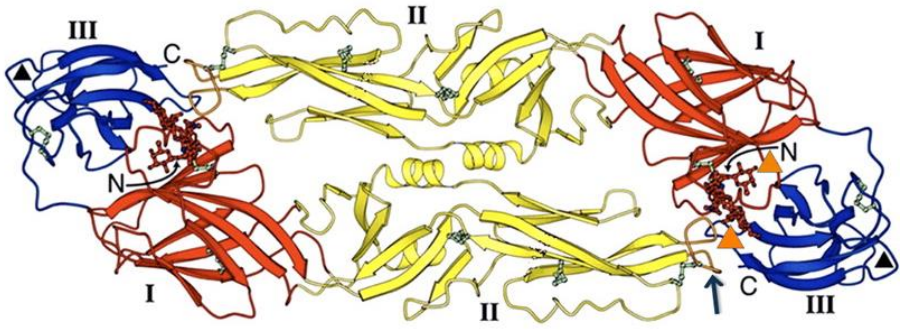


Fig. 2.2 E protein in dimeric form. The 180 subunits of E protein are clustered into 90 dimers on the surface of the virion which forms 60 trimers of post-fusion. Domain I, II and III are labelled in *red, yellow* and *blue* respectively. The arrow point indicate the fusion loop present in one subunit, while the orange triangles denote the putative conserved His residues on fusion peptides and the black triangle depict antibody-binding site (adapted from Harrison *et al.*, 2008) [41].

E protein domain III induces specific neutralizing antibodies in the host. The domain contains both linear and conformational epitopic regions [42]. These epitopes interact with target cell surface receptors (in case of KFDV it is not yet identified). It appears to be involved in low-affinity interactions with negatively-charged glycosaminoglycans on the host cell surface [37]. The interface between DI and DIII gets destabilized by the acidic pH milieu of the late endosome. The exact mechanism of viral entry to the host particularly that in KFDV is still unknown. Whether the viral entry follows membrane fusion path through clathrin-mediated or clathrin-independent mechanism, it is still to be uncovered.

2.2 Role of Domain III (EDIII) in inducing host immune response

The previous studies have shown that EDIII contains linear epitopic sites of high antigenicity targeted for virus neutralization [43]. So it is possible that for the development of differential diagnostic assays, EDIII variable epitopes can uniquely play a major role. To find out, multiple sequence alignment amino acids of KFDV-E with other closely related TBFVs such as TBEV-E and LIV-E was done to compare the variability among the sequences. The TBEV-E shares 80.85% whereas LIV-E shares 79.23% sequence [28] similarity with KFDV-E.

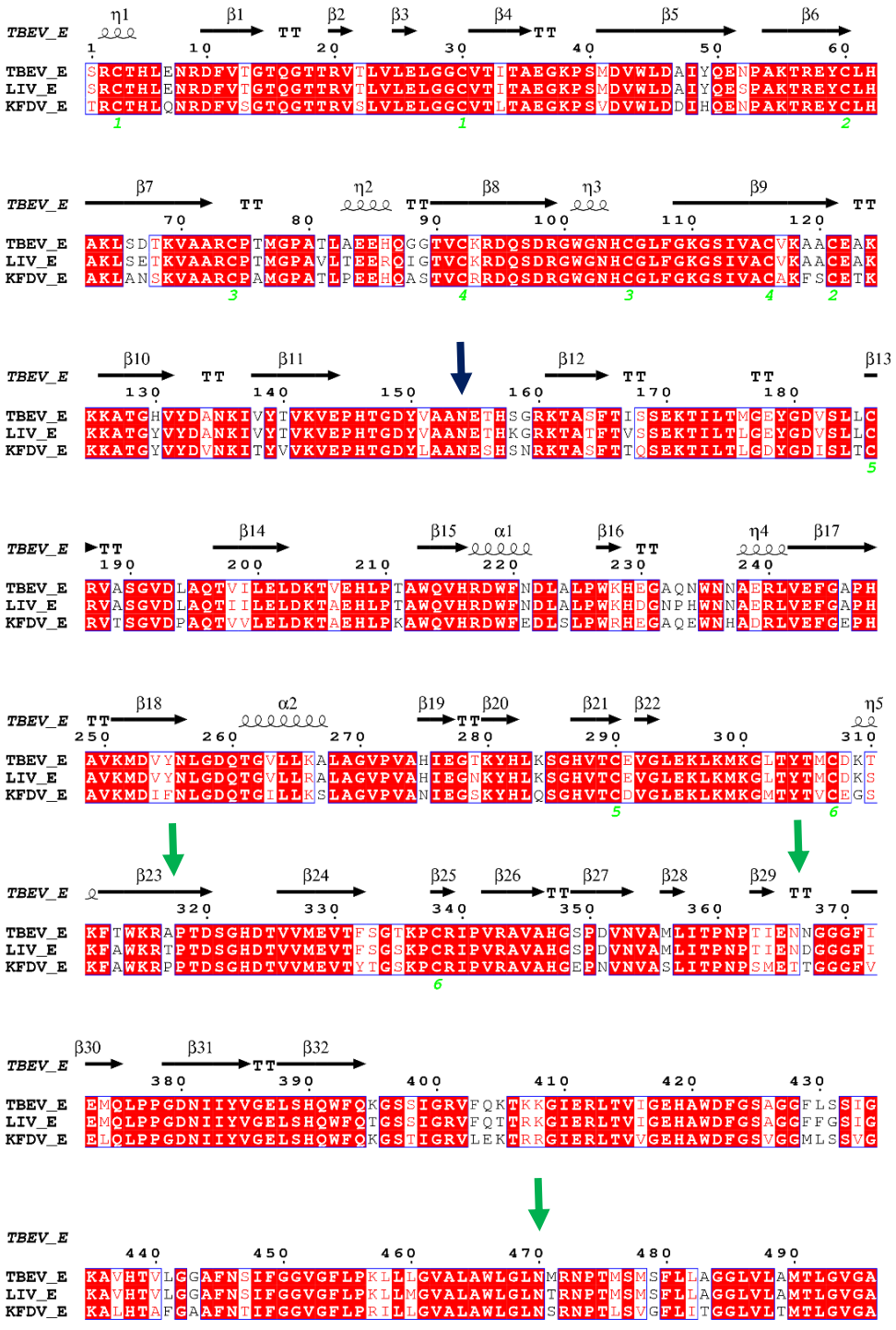


Fig. 2.3 Comparative sequence alignment of KFDV-E, TBEV-E and LIV-E. Sequences of TBEV-E (PDB: 5O6V) have been taken as reference for secondary structural elements. *Axis* represents β-strands, *helix* means α-helix, *TT* are the turning residues of β-strands. Numbers in

green points cysteine-cysteine disulphide bond formation. Down blue arrow indicates putative conserved site of glycosylation and down green arrows indicate varied residues falling in EDIII of the proteins. The alignment map was generated using ESPript 3.0 [44] and modified manually.

In TBEV, and in general flaviviruses, the EDIII forms the part of C-terminal comprising of ~100 amino acids. It is known as immunoglobulin-like domain due to its β-barrel structure composing of 6-10 anti-parallel β-strands [45]. Most of the flavivirus differ in the putative neutralizing epitopic sites on the EDIII [46]. Fig. 2.3 suggests putative epitopic residues present in KFDV-E when compared with closely related TBEV-E and LIV-E.

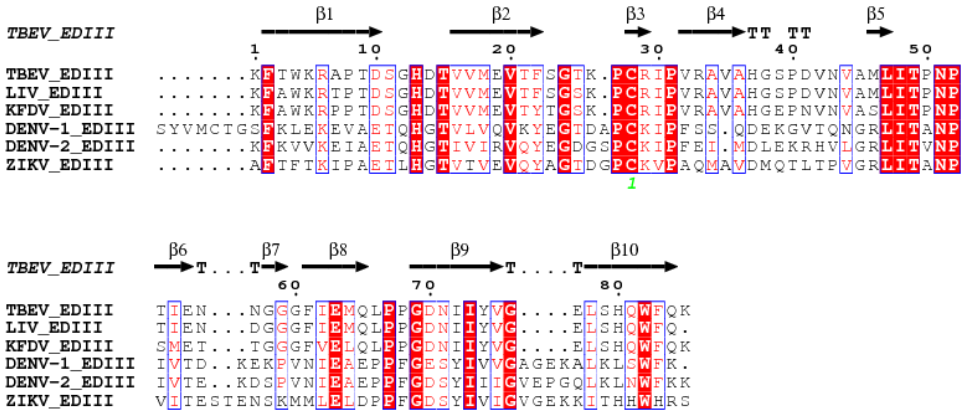


Fig. 2.4 Sequence alignment of EDIII domain of KFDV, TBEV, LIV, DENV1, DENV2 and ZIKV. TBEV-E (PDB: 5O6V) was taken as reference for secondary structural elements. Multiple alignments show conserved motifs in EDIII of both TBFVs and MBFVs responsible for antibody cross-reactivity. However, slight differences in amino acid also contribute to virus-specific epitopes and can be used for differential diagnosis. The alignment map was generated using ESPript 3.0 and further modified manually.

The serologic diagnosis of these flavivirus becomes challenging due to extensive cross-reactivity of antibodies arising from the conserved motifs especially in areas where two or more flaviviral infections are endemic. It is proposed that epitopic region of EDIII having signature sequences could be a target for virus-specific diagnosis approach. Hence, the recombinant expression of such epitopes followed by quality antibodies generation could be a potential approach for differential diagnosis of flaviviral infections [47]. ***I therefore hypothesize that if we target EDIII domain for antibody production, that antibody will precisely detect KFDV and no other flaviviruses (such as DENV, or ZIKV or TBEV) those commonly present in clinical cases in the endemic area.***

Thus, to test this hypothesis and to confirm specificity of anti-KFDV-EDIII antibodies we planned our experiment with following specific objectives.

I. To express recombinant KFDV-EDIII protein in *E. coli*.
II. To generate anti-KFDV-EDIII antibodies in rabbit.
III. To check the cross reactivity of anti-KFDV-EDIII antibodies with closely related viral antigens endemic to India (DENV serotypes, ZIKV etc.).

Similarly we also planned to check whether monoclonal antibodies of other flaviruses could potentially neutralize the KFDV EDIII. Taking bioinformatics approach, we checked protein-protein interaction with KFDV E protein as reported in the literature. For this, we used structural information of neutralizing antibodies of TBEV [48], WNV [49], DENV 1-4 [50] and JEV [51] for *in silico* analysis of putative binding sites on the backbone of KFDV-E protein. Since there is no solved E protein structure available in the PDB for KFDV E protein, the protein structure was predicted using a web-based server known as *i-tasser* (Iterative Threading ASSembly Refinement) [52] by utilizing the RefSeq available in NCBI

(accession: NC_039218) database. The aim is to know the protein structure prediction and determine spatial location of every atom from already available amino acid sequence using computational calculations. In *i-tasser*, the structural templates are identified from PDB by multiple threading approach in a hierarchical method [53].

The *i-tasser* assembly returned a single model with c-value (confidence score) of 2. The server estimated TM-score of 0.99±0.04 and RMSD of 3.1±2.2Å for the modelled KFDV-E protein. The c-score is the confidence score for estimating the quality of predicted models and values range in a linear scale from -5.0 to 2.0 with greater value indicating higher confidence in predicted structure. The TM-score is a metric for measuring the similarity of two protein structures and quantitatively is more sensitive to global topology (Fig. 2.5) and deviation.

In the same way, when EDIII domain structure was compared independently, structural similarity was observed betweem KFDV and TBEV like that of whole E protein. Predicted KFDV-EDIII had c-score of 0.99, TM-score of 0.85±0.08 and RMSD value of 2.1±1.7 Å. Modelled KFDV-EDIII distinctively showed 6 anti-parallel β-barrel sheets.

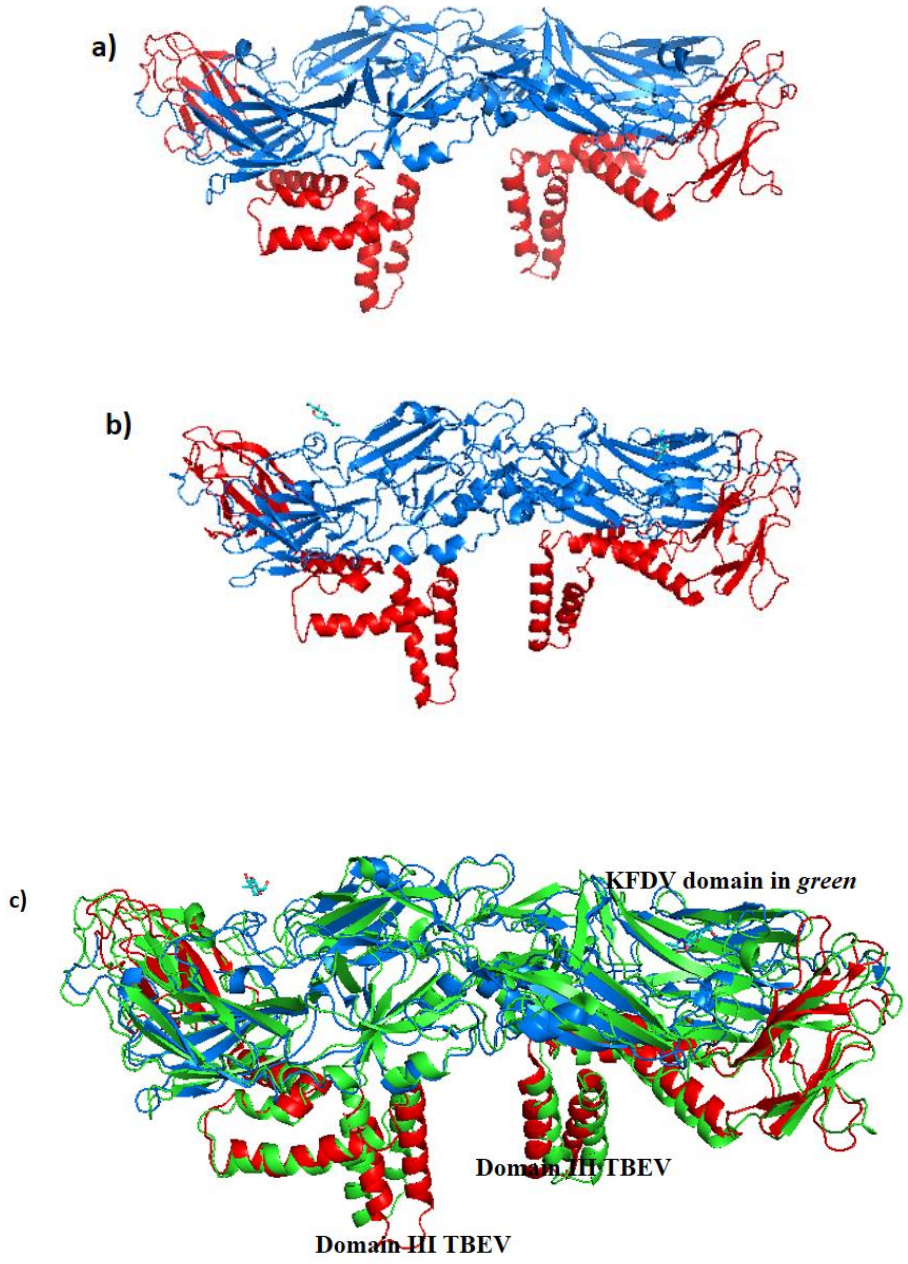


Fig. 2.5 *In-silico* comparison of KFDV E protein with that of TBEV. a) Modelled KFDV E using *i-tasser* having RMSD score of 0.99. b) TBEV E dimeric structure available in PDB: 5O6V. c) Superimposed structure of both the E proteins with RMSD of 2.13 Å. The symmetry monomer in each case was generated using PyMol and manually modified further. In Fig a) and b) EDIII are highlighted in red whereas EDI-EDII are in blue. The RMSD value indicates structure prediction close to TBEV.

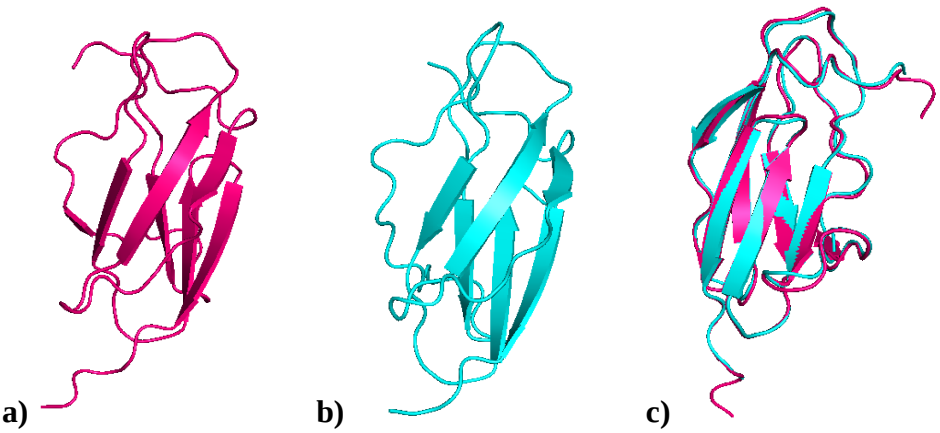


Fig. 2.6 *In-silico* analysis of KFDV-EDIII with TBEV-EDIII. a) The cartoon show modelled KFDV-EDIII, b) TBEV-EDIII (PDB: 5O6V). c) Structural alignment/ superimposition revealed the RMSD of just 0.33 Å. This suggests a close homology between the predicted structures and the already available structures. The structural alignment was done using PyMol tool.

Based on computational modeling of KFDV-E and KFDV-EDIII, it appears that close structural homology exists between structures of flavivirus E proteins (TBEV and KFDV), and therefore the antibody neutralization pattern would be similar.

2.3 Antibody mediated neutralization of flavivirus.

Antibodies employ several mechanisms to exert their antiviral effects. One is the capacity to directly bind to the incoming virus and neutralize the infectivity [54]. The other is by utilizing the effector functions of the Fc region of the antibody heavy chain [55]. There are a number of theories about the neutralizing effects of antibodies. One of the most widely accepted is the concept of "Single-hit" [56]. According to this model, antibody binding to functionally relevant sites on the virion is sufficient to disrupt viral entry and could abort viral infection process. Thus, antibodies bound to non-critical sites may not contribute to neutralization.

Since KFDV shares great sequential, structural and functional similarity with TBEV, I hypothesized that, like TBEV, KFDV-EDIII would also be the target for antibody-neutralization. To test this hypothesis, *in-silico* analysis using protein-protein docking was carried out. For this, a web-based server, ClusPro, was used [57] which is a template based docking method and is an example of rigid-body docking [58].

Docking was carried out using TBEV specific mAb 19/1786 [59] and the structure was then compared with KFDV-E protein dimer. Surprisingly, the monoclonal antibody showed neutralization to the same epitopic sites on EDIII as that on TBEV-EDIII (Fig. 2.7).

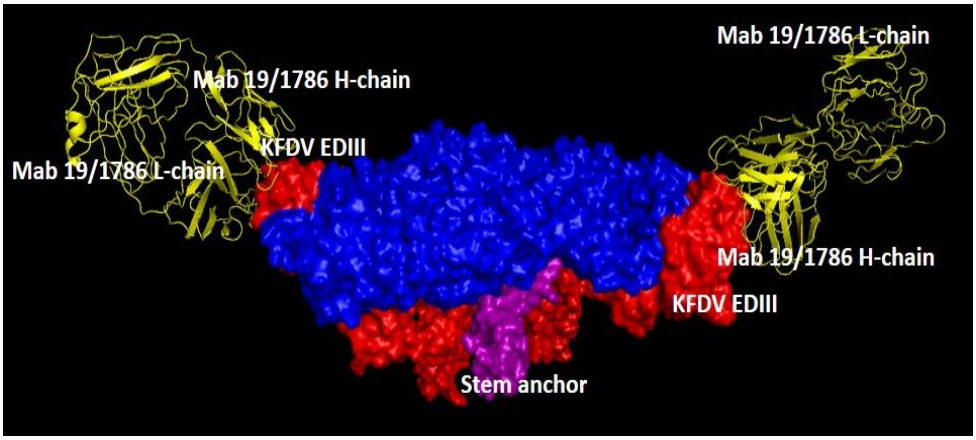


Fig. 2.7 Protein-protein docking of KFDV-E with mAb 19/1786. This suggests that other flaviviral monoclonal antibodies could cross-react with KFDV-EDIII. Thus, they could be used for therapeutic purposes but not for diagnostic purposes. The docking was done using ClusPro and manually modified using PyMol.

2.4 Antigen-antibody based rapid detection kit.

Antigen-antibody based diagnostic kit utilizes specificity of single antigen-antibody interaction mechanism. In this principle, enzyme-linked immunosorbent assay (ELISA) is developed to identify unknown antibodies or antigens present in the body fluid sample. In this technique, the antibodies are tagged with certain enzymes (proteins that speed up chemical reactions in the body). When an antigen binds to it respective antibody bind, the enzyme causes a reaction that produces a color change, thereby identifying the signature sequences present in unknown sample.

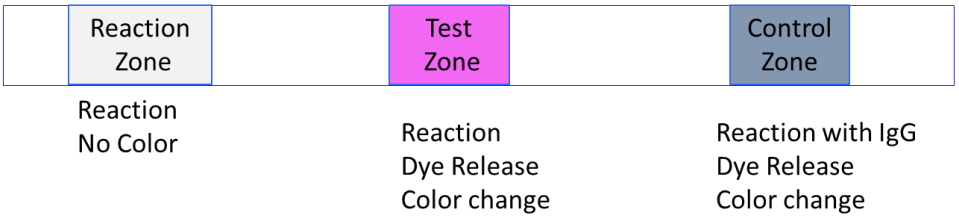


Fig. 2.8 Mechanistic principle of a lateral flow assay (LFA). In the case of infection with KFDV, E protein will function as antigen.

Based on the same exact principle, lateral flow assay (LFA) based kits are being commonly used for detection of antigen. These are simple, affordable point of care devices that can be used at end-user level without involvement of sophisticated infrastructure and skilled man power. Thus this point-of-care devices come with several advantages of being cost-effective, portable, reliable and rapid diagnostic method requiring minimum skills [60]. In this project, we attempted to design to produce LFA-based KFDV detection kit by utilizing KFDV-EDIII domain specific antibodies.

Chapter 3

Materials and Methods

3.1 MATERIALS

3.1.1 Media components and chemicals

Glutathione reduced, L-glutathione oxidized, PMSF, BSA, Tris-free base, EDTA, agarose low EEO, D-(+)-glucose, yeast extracts and urea were procured from HiMedia (Mumbai, India). Tetracycline hydrochloride, ampicillin sodium salt, D-biotin, IPTG, DTT, chloramphenicol, glycine, acrylamide, L-arginine (free base), sodium chloride, bis-tris and isopropanol were procured from Sisco Research Laboratories (SRL) Pvt. Ltd (India). Adenosine 5′-triphosphate disodium salt hydrate, ammonium persulphate (APS), Ponceau S, Potassium phosphate monobasic, imidazole, bromophenol blue, dimethyl sulphoxide, Tween-20, triton X-100 and sodium hydroxide were procured from Merck Sigma Aldrich. Agarose and chloroform were procured from MP Biomedicals (Mumbai, India) whereas bis-acrylamide was procured from Promega (Delhi, India). Sodium dihydrogen phosphate, acetic acid glacial (99-100%) and acetone were procured from Merck. Serva Chemiluminescence Reagent for Horseradish Peroxidase was used for visualization of fluorescence in western blot. Hydrogen peroxide was procured from Finar Limited. FavorPrep^{TM} GEL/PCR Purification Kit was used for gel extraction protocols.

3.1.2 Molecular weight standards

For estimating the molecular size of DNA during agarose gel electrophoresis experiments, 1Kb DNA ladder and 100bp DNA ladder was procured from GeneDireX Inc (India). For determining the size of the protein, commercial standard molecular weight ladder (Precision Plus

Protein™ Dual Color Standards) was procured from BioRad (Gurgaon, India) and Prestained Protein Ladder from HiMedia (Mumbai, India).

3.1.3 Dialysis tubes

Molecular porous cellulose dialysis membrane tubing's of different molecular weight cut off ranging from 6000-14000 were procured from Sartorius Pvt. Ltd (Hyderbad, India).

3.1.4 Primers (oligonucleotides)

The DNA primers for this work were synthesized at Eurofins Genomics India Pvt Ltd, Bangalore, India.

3.1.5 Chromatography columns

Ni Sepharose™ 6 Fast Flow beads for purification of His-tagged proteins were obtained in un-packaged slurry from GE Healthcare, USA. Pre-packaged HisTrap™ HP column of 1 ml and HisTrap™ HP column 5 ml as well as Superdex 200 10/300 columns were also obtained from GE Healthcare.

3.1.6 Enzymes

Taq DNA Polymerase and site-specific *Tobacco etch virus* (TEV) protease were prepared in-house. *Phusion* Polymerase was purchased from ThermoFisher (Mumbai, India). All type-II restriction endonucleases (BamHI, HindIII, BsrgI, EcoRI) and T4 DNA ligase were acquired from New England Biolabs (USA). Lysozyme (chicken egg white) was purchased from MP Biomedicals, India. Calf intestinal alkaline phosphatase (CIP) and RNaseA (bovine pancreas) were procured from Merck Sigma Aldrich.

3.1.7 Instruments

PCR amplification was done in Thermocyclers of Applied Biosystems, Thermo Fisher Scientific (ProFlex™ PCR System). Beckman Coulter Optima XPN-80 ultracentrifuge was used for rotein purification and high speed varying capacities Eppendorf centrifuge 5418 R and Sorvall ST 8R centrifuge (ThermoFisherScientific) were used for nucleic acid work. For electrophoresis visualization, the gel documentation system (ImageQuant LAS 4000) by GE Healthcare Life Sciences, and for bacterial growth REMI CIS-24 Plus Orbital Shaking incubator was used. Ultra-sonic cell disruptor (Probe sonicator, QSONICA Sonicators) were used for protein purifciation. For nucleic acid electrophoresis, stander midi submarine electrophoresis unit of local made and for protein electrophoresis and SDS-PAGE (Mini Dual Vertical Electrophoresis Unit) from Tarsons was used. Protein purification was exclusively done with ÄKTA™ pure instrument set at 4℃.

3.1.8 Preparation of liquid reagents used in this work

The following end user reagents were made in house with help of individual ingredients purchased from various sources. The formula of such reagents are described below.

2xTY (broth media)

1.6% bacto-tryptone	16 g bacto-tryptone
0.5% NaCl	5 g NaCl
1.0% Yeast extract	10 g Yeast extract

Volume made up to 1 liter with MiliQ water and autoclaved at 121℃ at 15 psi for 15 min.

TYE Plates

0.5% Yeast Extract	5 g Yeast extract
0.8% NaCl	8 g NaCl

1.5% Agar 15 g Agar

Volume made up to 1l with MiliQ water and autoclaved at 15 psi at 121°C for 15 min.

Antibiotics

1. **Ampicillin (sodium salt**): Working stock solution (1,000X) of antibiotics (50 mg/ml) in sterile distilled water was prepared and stored at -20°C. The final concertation was adjusted to 50 µg/ml in liquid media and 100 µg/ml in agar plates.

2. **Chloramphenicol:** Stock solution 34 mg/ml (1,000X) was initially prepared in absolute ethanol and stored at -20°C. The final concertation was adjusted to 34 µg/ml in media and plates.

Reagents for plasmid isolation

- **Bacteria resuspension solution**

  25 mM Tris-Cl pH 8.0

  50 mM glucose

  10 mM EDTA-Na

Filter through 0.45 µm syringe filter and store at 4°C.

- **Bacteria lysis solution (NaOH/SDS/ TENS)**

  10mM tris-HCl

  1mM EDTA

  1.0 % SDS

  0.2 M NaOH

The solution was stored at room temperature for future use.

- **Neutralizing buffer**

  2.5 M Glacial acetic acid (from stock of 17.5M glacial acetic acid)

  5.0 M potassium acetate

Filter through 0.45µm syringe filter and store at 4°C**.**

- **Phenol: chloroform: Iso-amyl alcohol**

  Equilibrated chloroform, phenol, and isoamyl alcohol are added in the respective volume ratios (25:24:1).

- **RNase A 10 mg/ml**

  10 mM NaCl 10 µl of 5 M NaCl

  RNase A 50 mg

Volume was made-up to the total of 10 ml with MiliQ water. Boiled for 15 min, slowly allowed to cool to RT and store at -20˚C.

- **TE (10:50)**

  50 mM EDTA

  10 mM Tris-Cl

Prepared with MiliQ water

- **TE (10:1)**

  1 mM EDTA

  10 mM Tris-Cl

Prepared with MiliQ water

- **TE (10:0.1) (250ml)**

  0.1 mM EDTA

  10 mM Tris-Cl

Prepared with MiliQ water

- **3 M Sodium acetate**

  62.2g sodium acetate salt.

  pH 5.2 was adjusted with 1.0 M NaOH.

Volume adjusted to 250 ml with MiliQ water

Reagents for agarose gel electrophoresis

- **Ethidium bromide**

  10 mg/ml (15 mg EtBr in 1.5 ml MQ water)

- **6x DNA Gel loading Dye**

Glycerol	3 ml (100%)
1 M Tris-Cl	60 µl
0.5 M EDTA	600 µl
Bromophenol blue	15 mg
Xylene cyanol	15 mg

Prepared with 10 ml of MiliQ water.

- **50x TAE buffer (1 l)**

Tris free base	242 g
Disodium EDTA	18.61 g
Glacial acetic acid	57.1 ml

Volume was made up to 1l with MiliQ water.

Reagents for SDS-PAGE

- **15% resolving/ separating (10 ml)**

MiliQ water	3.75 ml
30% acrylamide/0.5% bisacrylamide	5.0 ml
3 M Tris-Cl pH-8.8	2.5 ml
10% SDS	100 µl
TEMED	10 µl
25% AMPS	40 µl

- **5% stacking gel (5 ml)**

MiliQ water	1.225 ml
10% Acryl amide, 0.5% Bisacrylamide	2.5 ml
0.5 M Bis-Tris pH-6.8	1.2 ml
10% SDS	90 µl
TEMED	4 µl
25% AMPS	20 µl

- **5x gel running buffer (2 l)**

Tris Base	60.56 g
Glycine	288 g
SDS	10 g
Distilled water	2 l

- **Fixing and de-staining solution (for 1 l)**

Methanol	400 ml
Glacial acetic acid	100 ml
Distilled water	500 ml

- **2x PGLB (Protein Gel Loading Dye)**

0.5 M Bis-tris pH 6.8	2.5 ml
100% Glycerol	2.0 ml
10% SDS	4.0 ml
β-mercaptoethanol	1.5 ml
Bromophenol blue	4 mg

β-mercaptoethanol was not added while preparing the stock of PGLB rather added at the time of usage.

- **Staining solution (250 ml)**

Coomassie Brilliant Blue R-250	0.75 g
Glacial Acetic acid	25 ml
Methanol	100 ml
Distilled water	125 ml

Reagents for protein preparation and purification

- **IPTG (Isopropyl β-D thiogalactoside)**

  0.84 M IPTG stock (200 mg/ml IPTG in MiliQ water). Stored at -20°C.

- **2 M Imidazole**

  272.32 g of imidazole per liter of MiliQ water with pH adjusted to7.0 with HCl. Filtered through 0.45 µm syringe filter and stored at 4°C**.**

- **5X P-300 (1 l)**

Na_2HPO_4	21.65gm
$NaH_2PO_4.2H_2O$	15.21gm
NaCl	87.66gm

Volume adjusted to 1 l with MiliQ water.

- **1xP300 IM500 (1 l)**

1x P300	200 ml (5x P300 stock)
500 mM Imidazole	250 ml (2M Imidazole stock)

Volume adjusted to 1 l with MiliQ water.

- **T200 (1 l)**

20 mM Tris-Cl	200 ml (1 M Tris-Cl stock)
200 mM NaCl	25 ml (5 M NaCl stock)

Volume adjusted to 1 l with MiliQ water.

Reagents for Western blotting

- **Transfer buffer (for 2 l)**

Glycine	28.8gm
Tris base	6.06gm
Methanol	400 ml
Autoclaved MiliQ water	1600 ml

Methanol was added at the time of usage and the volume was adjusted to 2 l.

- **10X TBS (for 1)**

Tris base	24.2 g
NaCl	80 g

pH was adjusted to 7.6. Autoclaved at 15 psi at 121°C for 15 min and stored in the room temparature.

- **1X TBS (For 1l)**

  100 mL of 10X TBS in 900 ml of autoclaved MiliQ water.

- **1X TBST**

  0.1 % Tween 20 in 1X TBS

  1ml of Tween 20 in 1000 ml 1X TBS

- **5% Blocking solution (For 20 ml)**

1X TBST	20 ml
Non-fat milk powder	1g

- **Ponceau S stain (For 30 ml)**

(Ponceau Stain: 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid)

Autoclaved MiliQ water	10 ml
Glacial acetic acid	0.3 ml
Ponceau S	0.033 g

Volume adjusted to 30 ml with MiliQ water and store at room temperature.

- **H_2O_2**

  Aliquots of one ml was prepared and wrapped with aluminum foil (light sensitive) and stored in the dark.

Reagents for protein denaturation and refolding

- **Inclusion bodies wash buffer (1 l):**

20mM Tris-Cl pH 8.0	20 ml from 1M Tris-Cl
0.5mM EDTA-Na_2	1 ml from 0.5M EDTA stock
100mM NaCl	20 ml from 5M NaCl
10 mM β-mercaptoethanol	0.7 ml from 14.4 M stock

Volume adjusted to 1 l with MiliQ water.

- **Triton wash buffer**

20mM Tris-Cl pH 8.0	16 ml from 1M Tris-Cl
0.5mM EDTA-Na_2	0.8ml (0.5M EDTA)
100mM NaCl	16ml from 5M NaCl
10mM β-Mercaptoethanol	0.56ml from 14.4 M stock
1% Triton X-100	8ml from 100% stock

Volume adjusted to 1 l with MiliQ water.

- **Tris Urea IM 40 (TUI40) (100 ml)**

20mM Tris-Cl pH-8.0	2 ml (1M stock)
200mM NaCl	4 ml (5M stock)
8M urea	48 g
40mM imidazole	2 ml (2M stock)

Volume was adjusted to 1 l with MiliQ water.

- **Tris Urea IM 750 (TUI750) (100 ml)**

20mM Tris-Cl pH-8.0	2 ml (1M stock)
200mM NaCl	4 ml (5M stock)
8M urea	48gm
750mM imidazole	38 ml (2M stock)

Volume was adjusted to 1 l with MiliQ water.

3.1.9 Details of experimental hosts

Table 3.1: Details of experimental host

BL21(DE3)pLysS *(E. coli)*	Expression host	*E.coli*, BF^- dcm, Ompt, hsds(lb-mb), galλ(DE3)
		Ompt-Mutation in outer membrane of protease VII reducing proteolysis of expressed protein
		DE3-lysogen encoding T7 RNA polymerase that is used to induce expression in T7-promoter based expression system.
		$rB/K^{+/-}$ -B/K defines the strain lineage.+/- indicates whether strain got restriction system.

		MB/$K^{+/-}$ -Defines strain lineage indicates whether strain has the restriction system

DH5α (*E. coli*)	Cloning host	E-ColirecA1,gyrA 96,relA/thi-1,supE44,hsdsR17,Δ lac U169(φ 80 lac z Δ M15)
		The bacterial genome lack functional restriction and methylation activities.
		Chromosomal deletion of gene between the listed gene
		Mutation in DNA gyrase conveys nalidixic acid resistance
		Partial deletion of the LacZ gene that allows α complementation of β Galactosidase gene required for blue/white selection on X gal plates; deletes amino portion of LacZ (amino acid 11-41)
		For reduced occurrence of unwanted recombination in cloned DNA the strain is UV sensitive and deficient in UV repair.
		Relaxed phenotype, permits RNA synthesis in absence of protein synthesis
		Requires thymine

3.1.10 *E. coli* expression vectors used in the study.

The expression vectors used in the study are the modified form of pST44 poly-cistronic expression systems [61].

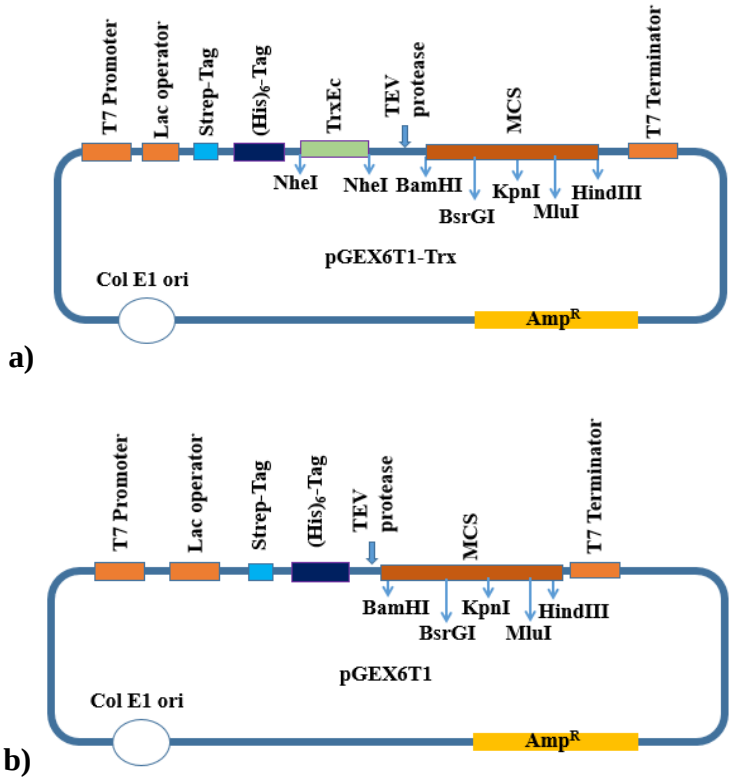


Fig. 3.1 a) Plasmid construct pGEX6T1-Trx, and b) Plasmid construct pGEX6T1 (without Trx). In both cases the parent pST44 vector was modified with insertion of N-terminal 6xHis-tag used for immobilized metal affinity purification of the recombinant proteins. Major role of trx was to increase solubility and to promote reduced environment in the cell for proper folding of recombinant proteins.

Both the vectors contain pMB1 and ColE1 origin of replication and T7 promoter and terminator sequences. They also contain N-terminal small peptide fusion tags, as streptavidin-6x His-TEV protease cleavage site, used for efficient purification of recombinant proteins. The two plasmids (pGEX6T1 and pGEX6T1-Trx) differ by the presence of 12 kDa *E. coli* TRX-tag placed between His6x-tag and TEV protease site (Fig 3.1). The

thioredoxin (TRX) tag is mainly used to increase the solubility of the proteins being expressed in the bacterial systems. Also, since bacterial systems like *E. coli* are deficient in chaperones, presence of TRX-tag increases the thermal stability and helps in refolding of proteins requiring reducing environment [62].

3.1.11 Bioinformatics and statistical analysis

For the gene synthesis of envelope protein domain III of KFDV, DENV1, DENV2, DENV3, DENV4 and ZIKV, the DNA sequences were optimized for codon usage to get maximum expression of protein in the prokaryotic system. For this, an online server, *OPTIMIZER* [63] was used. Primer designing for PCR, construct preparation and other cloning related work was virtually carried using the Serial cloner version 2.6.1 software. Several of NCBI tools were utilized as NCBI-Pubmed, BLAST, NCBI-nucleotide and NCBI-protein. *BioEdit* version 7.0.5.3 was utilized for DNA sequence alignment and sequence analysis. Clustal Omega [64], an online tool, was used for multiple sequence alignment. *Espript 3.0* [44] server was used for sequence and structure based alignment of multiple proteins. The three-dimensional structure of protein molecules was predicted using amino acid sequence, a web-based server, *i-tasser* [53] was used. Protein-protein docking studies were carried out using an online tool, *ClusPro* [58]. Molecular graphics and analysis were performed using PyMOL [65].

3.2 METHODS

3.2.1 KFDV envelope protein-DIII CDS synthesis

KFDV-EDIII CDS (97 amino acids) of the envelope glycoprotein E was synthesized in-vitro using primer overlapping gene synthesis method [66]. The technique utilizes the features of both dual asymmetric PCR [67] and overlap extension PCR method [68].

For CDS synthesis of EDIII protein, overlapping primers were designed and optimized based on expression host. The KFDV E protein gene sequence was codon optimized taking consideration of available sequence at GenBank-NCBI (accession number JF416958.1) such that EDIII can be cloned with BamHI restriction site at the 5' end while HindIII restriction site at 3' end of the CDS. About 3 bp flanking sequences ware added at both ends to facilitate efficient binding and processing by the restriction enzymes (see Table 3.2). The codon optimization was *E. coli* specific.

The dual asymmetric and overlapping extension PCR was performed using long-stretched oligonucleotides with overlapping bases in a two-step process (Fig. 3.2). Briefly, two separate PCRs were set-up using a primer pair; ODN 78-79 and ODN 80-81 having 18 bp and 12 bp overlap respectively. In the first round of amplification, CDS of 177 bp and 145 bp respectively were synthesized. The final round of PCR was carried out using products of PCR1 and PCR2 (having 12 bp overlap) as templates and this was extended to the desired size of 310 bp.

Table 3.2: Oligonucleotides for gene amplification of KFDV-EDIII

Primer	**Primer Sequence (5'→3')**
ODN 78 (Forward) BamHI site	CGT**GGATCC**CCGTATGTGAGGGTTCAAAGTTTG CCTGGAAACGTCCACCTACGGATAGTGGTCACG ACACAGTTGTAATGGAGGTCACATATACCGGTT CCAAGCCT
ODN 79 (Reverse)	TGGCGTAATCAGTGATGCTACGTTGACGTTTGG CTCTCCGTGAGCCACTGCCCTTACTGGGATTCT ACAAGGCTTGGAACCGGTATA
ODN 80 (Forward)	CTGATTACGCCAAACCCTTCAATGGAAACAACT GGTGGAGGATTCGTTGAACTGCAGTTGCCT
ODN 81 (Reverse) HindIII site	CGT**AAGCTT**ACTTCTGAAACCATTGGTGTGATA ATTCACCGACATATATGATGTTATCACCTGGAG

	GCAACTGCAGTTCAAC

Table 3.3: Thermal cycling conditions of PCR

Step		**Temperature**	**Time (mm:ss)**
Initial denaturation		95ºC	00:30
Amplification (35 cycles)	Denaturation	95ºC	00:10
	Annealing	~55-65°C	00:20
	Extension	72ºC	00:30-01:00
Final Extension		72ºC	05:00
Hold		4ºC	∞

The reaction mixture for PCR extension and amplification comprised of 1×*Phusion* reaction buffer pH 8.8, 1.5 mM dNTPs, 150-200 nM primer pairs and 0.5 units *Phusion* DNA polymerase. DMSO was also added to reduce chances of secondary structure formation. The PCR run program was set as mentioned in table 3.3. Finally, the PCR products were analysed by agarose gel electrophoresis.

Ethidium bromide as nucleic acid stain was used to visualize the final PCR products in agarose gel electrophoresis. After getting the expected band size of 310 bp, the PCR clean-up protocol was followed to remove PCR components.

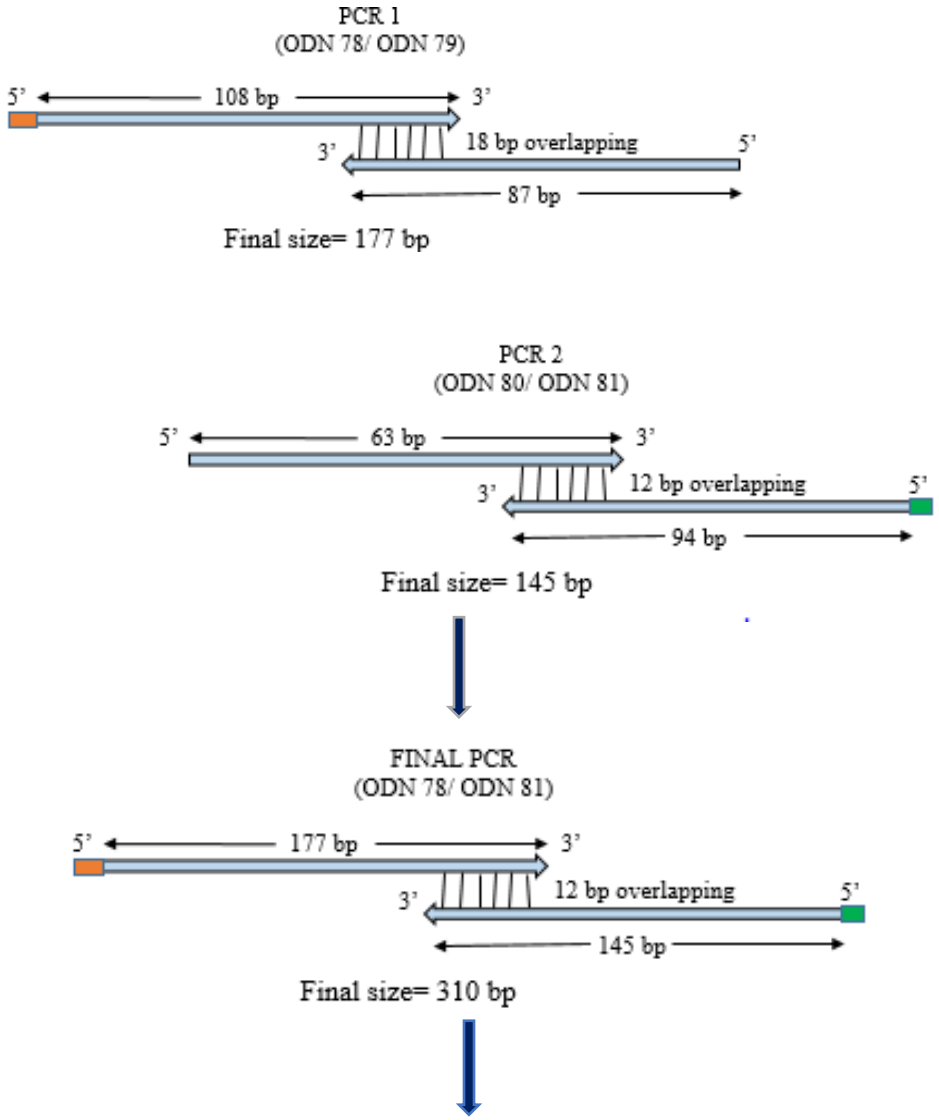


Fig. 3.2 KFDV EDIII gene synthesis strategy. Two separate overlap extension PCR were set up. The final asymmetric PCR amplification was obtained using end primers adding BamHI-HindIII sites for downstream cloning.

Summarily, the PCR pooled products were made up to a volume of 200 µl using TE (10:0.1) buffer. About 2.5 folds phenol: chloroform: isoamyl alcohol mix in the ratio of 25:24:1 was added. The mix is then spun down at full speed (15,000 rpm) for 5 min. Upper aqueous layer was carefully collected and the traces of phenol was further removed by washing twice

with chloroform: isoamyl alcohol in the ratio 24:1. About 2.5 fold volume of absolute ethanol was used to precipitate DNA in the presence of 1/10th volume of 3M sodium acetate at pH 5.2 followed by 10 min incubation on ice and centrifugation at 15,000 g for 15 min.

The obtained pellet was washed with 70% ethanol, dried at 50°C and dissolved in 20 µl of TE (10:0.1) buffer and stored in -20°C for future use.

3.2.2 KFDV EDIII cloning in *E. coli* expression plasmids

Amplified and purified KFDV EDIII was cloned between BamHI and HindIII sites located in the downstream of T7 promoter. The protein cassette was translationally fused with Strep-His tag and Trx tag (pGEX6T1 and pGEX6T1-Trx) as depicted in Fig. 3.1.

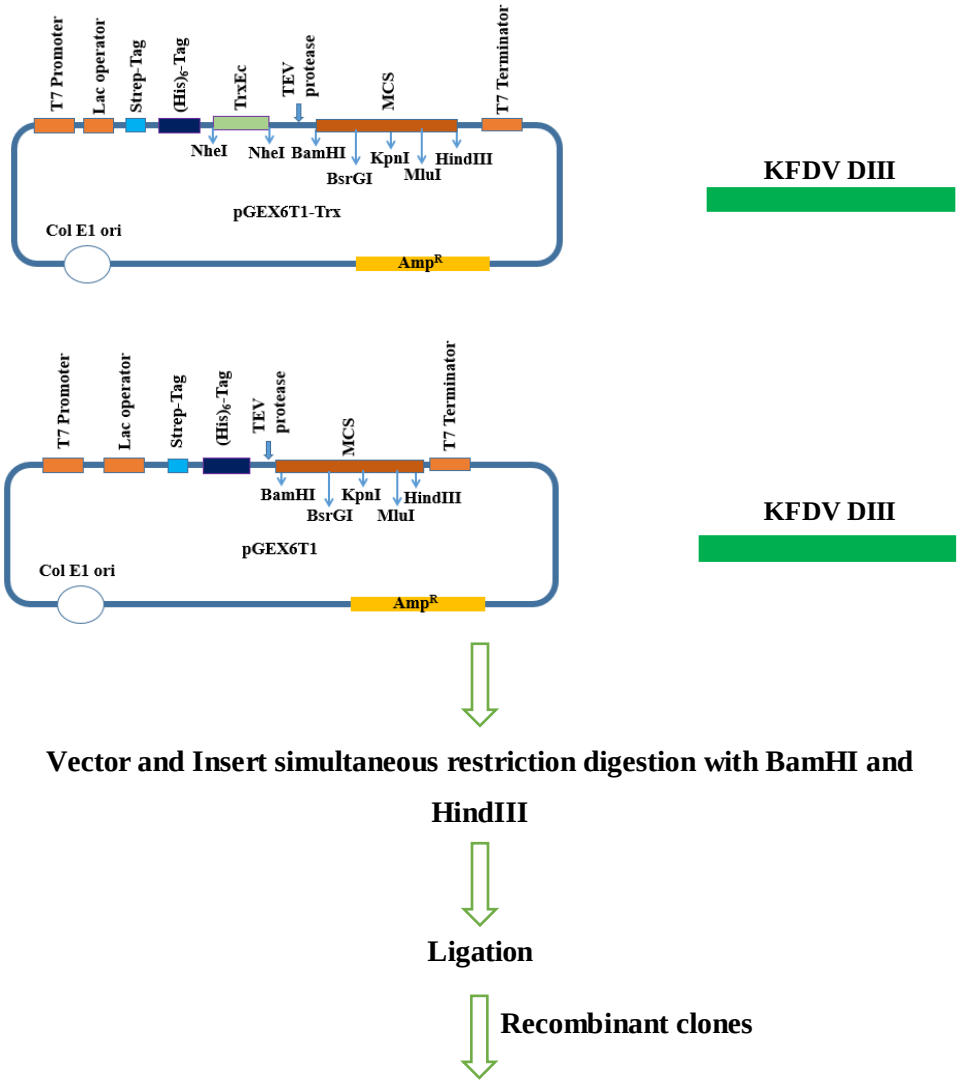
The optimal restriction enzyme digestion conditions like pH, metal cofactors, temperature and specific activities were provided in the supplier's manual (NEB). Both the vector and insert digested products were gel purified and subsequently used for ligation reaction. The ligation was performed in 3:1 molar ratio of insert and vector at 21°C for 1 hour using T4 DNA ligase. 2 µl of ligated mixture was transformed into *E. coli* DH5α competent cells kept on ice and subsequently heat-shock method was employed.

In outline, 100-200 ng of DNA was mixed with 100 µl of competent cells and incubated on ice for 30 min. Next, the cells were incubated at 42°C for 90 sec in water bath and instantly transferred to ice for 5 min. After incubation, volume was made up to 1ml using sterile 2×TY media and incubated at 37°C for ~1 hour with shaking at 200 rpm. The competent cell suspension or whole cells (settled down by centrifugation and re-suspended) were then plated on appropriate antibiotic containing plates and were incubated over night at 37°C. Typically, *E. coli* transformants in

the form of white shining colonies were observed on the plates 12-16 hour post incubation.

3.2.3 Colony PCR and confirmation of KFDV-EDIII cloning

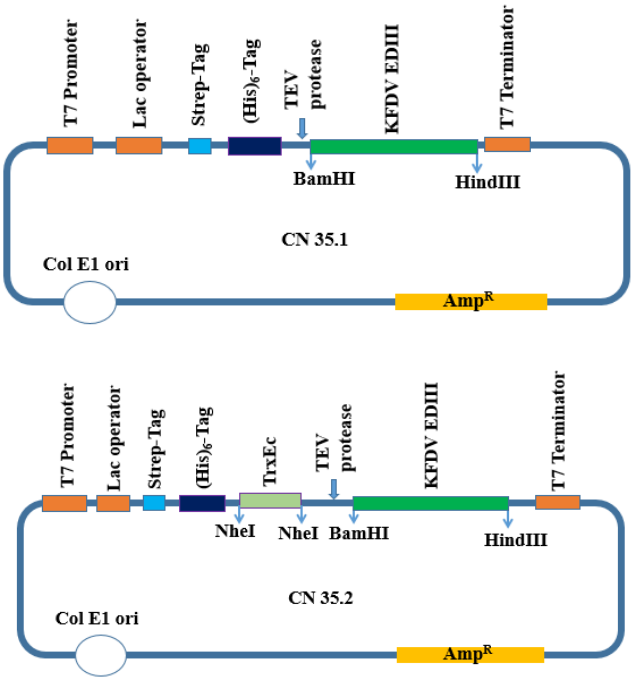
Recombinant clones were first screened by colony PCR technique, restriction digestion and further were confirmed by DNA sequencing. Colony PCR was performed in the same manner of regular PCR, except, the DNA template was replaced by fresh bacteria colony picked from the transformed plate.



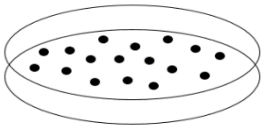
Vector and Insert simultaneous restriction digestion with BamHI and HindIII

Ligation

Recombinant clones



Transformation into *E. coli* DH5α cells by heat shock method and spread plate on agar plate containing 50 µg/ml ampicillin



Colony PCR for screening of recombinant clones

Plasmid (recombinant clone) purification

Fig. 3.3 Generation of recombinant clones. The clones 35.1 and 35.2 were generated by cloning KFDV-EDIII CDS into BamHI and HindIII sites with fusion His-tag at N-terminal.

3.2.4 Recombinant plasmid (clone) purification

Bacterial cultures inoculated with positive colonies confirmed earlier were grown overnight (14-16 hrs) with constant shaking at 37°C at 220 rpm. This was followed by plasmid purification by alkaline lysis method. In a nutshell, the overnight grown cultures were spun down at 6,000 rpm for 10 min at room temperature and the pellet was solubilized in re-suspension buffer followed by incubation in the lysis buffer (5 ml for midi prep). This lysed cells were subjected to twice volumes NaOH/SDS treatment, mixed gently and incubated at room temperature for 5-7 min. To precipitate chromosomal DNA, solution of potassium acetate and acetic acid is added and further incubated in ice for 5-7 min and the kept on ice. All cell debris is later removed by spinning at 15,000g for 20 min at 4°C. The cleared supernatant obtained was filtered through a sintered glass funnel. Finally, to precipitate the plasmid DNA, the supernatant was incubated with isopropanol at -20°C for 10-15 min before centrifugation.

After centrifugation at 13,000 rpm for 5 min at 4°C, the obtained plasmid DNA pellet was washed with 70% ethanol and later dissolved in 100-200 µl TE (10:50) buffer. This form of DNA was incubated with 5 µl of 10 mg/ml RNase A at 37°C for 30 min. To denature RNase, plasmid DNA was extracted once with 0.3 ml of PCI and twice with 0.5 ml of CI reagent. 2.5 volume of absolute ethanol was used to precipitate DNA in the presence of $1/10^{th}$ volume of 3 M sodium acetate pH 5.2 followed by incubation at -20°C for 20-30 min. The obtained DNA pellet was further washed with 70% ethanol, dissolved in TE (10:0.1) buffer and stored at -20°C.

3.2.5 Expression and immobilized metal affinity purification of recombinant KFDV EDIII

For overexpression of recombinant proteins, commonly used hosts are BL21 (DE3) pLysS cells which contain the chromosomal copy of the T7

RNA polymerase. This particular strain contains both DE3 lysogen and pLysS plasmid. pLysS constitutively expresses low levels of T7 lysozyme which reduces basal expression of recombinant genes by inhibiting basal levels of RNA polymerase (which in itself is under the control of lacUV5 promoter). This arrangement is present on the phage genome called DE3. T7 lysozyme by pLysS binds to T7 RNA polymerase causing inhibition until induction. pLysS contains p15A origin. This origin allows pLysS to be compatible with plasmids containing ColE1 or pMB1 origin derived plasmids.

Purified recombinant clones were transformed into competent BL21 (DE3) pLysS cells by heat shock method and recombinant colonies were screened on ampicillin and chloramphenicol plates. 10 ml of primary culture grown in 2xTY broth, supplemented with 50 μg/ml of ampicillin and 50 μg/ml chloramphenicol, was incubated for approx. 6 hours at 37°C at 220 rpm on an orbital shaker and subsequently inoculated into secondary culture in 1:100 dilution. Cultures were allowed to grow till OD_{600} reached to 0.8 at 37°C at 220 rpm. The cultures were induced with 0.2mM IPTG and incubated at 28°C for 18 hours. Culture was harvested at 7,000 rpm for 5 min and the cell mass was suspended in 30 ml of 1x P300 buffer. Then the cell pellet was stored at -80°C until further processing.

Cell pellet were thawed at 30°C for 30 min followed by sonication on ice at 75% amplitude with 3 sec on-off pulse cycle for 5 min. Lysed whole sample was spun down at 18,000 rpm for 15 min at 4°C. Supernatant was incubated with 500 μl of Ni Sepharose™ 6 Fast Flow beads (pre-equilibrated with 1xP300 IM20) at 4°C. Ni-sepharose beads present the principle of immobilized metal affinity chromatography in which affinity of nickel with 6xHis-tag is utilized for purification of recombinant proteins and peptides. Bound protein was eluted in 1.5 ml of 1xP300

IM500 buffer. At each step of protein purification, samples were collected for downstream analysis (SDS-PAGE) to monitor the protein expression and purity of the expressed protein. The procedure was scaled up to get large amounts (milligram quantity) of recombinant proteins.

Since maximum amount of protein was found in inclusion bodies, the pellet was further processed for denaturation by solubilizing and processed for refolding *in vitro*.

3.2.6 DENV (1-4) EDIII and ZIKV EDIII gene amplification and cloning

The envelope protein domain III of five other flaviviruses (DENV1, DENV2, DENV3, DENV4 and ZIKV) gene was gifted by Dr. Siddappa Byrareddy, Associate Professor, University of Nebraska Medical Center, Omaha, USA.

For gene amplification, primers were designed for the codon-optimized gene sequence based on the sequence information available at GenBank-NCBI. Table 3.4 lists the primer sequence used and the GenBank- NCBI accession number for sequence retrieval.

Amplified and purified EDIII CDS fragments were cloned between restriction sites in T7 promoter based expression plasmids (pGEX6T1-Trx) containing N-terminal Strep-His tagged fusion peptides as mentioned earlier for KFDV EDIII. Transformed DH5α cells were screened on ampicillin (50μg/ml) plates and later confirmed by colony PCR. Recombinant clones were purified, sequence verified and stored at -20°C until further use.

Table 3.4: Oligonucleotides for CDS amplification of DENV serotypes EDIII and ZIKV EDIII

Flavivirus (accession number)	Cloning sites	Primer Sequence (5'► 3')
DENV-1 (NC_001477)	BamHI (F)	CGT**GGATCC**AAAGGGATGTCATATGTAATGTG
	BsrGI (R)	CGT**TGTACA**TTAACTGCTTCCCTTCTTGAACC
DENV-2 (NC_001474)	BamHI (F)	CGT**GGATCC**AAAGGAATGTCATACTCTATGTG
	HindIII (R)	CGT**AAGCTT**AAGAACTTCCTTTCTTAAACCAG
DENV-3 (NC_001475)	BamHI (F)	CGT**GGATCC**ATGAGCTATGCAATGTGCACG
	HindIII (R)	CGT**AAGCTT**ACCCAATCGAGCTCCCCTTC
DENV-4 (NC_002640)	BamHI (F)	CGT**GGATCC**AAGGGAATGTCATACACGATG
	HindIII (R)	CGT**AAGCTT**AGGAACTCCCTTTCCTGAAC
ZIKV (NC_012532)	BamHI (F)	CGT**GGATCC**AGATTGAAGGGCGTGTCATATT
	HindIII (R)	CGT**AAGCTT**AACCACTCCTATGCCAGTG

3.2.7 Expression and affinity purification of recombinant DENV (1-4) and ZIKV EDIII

For overexpression of recombinant proteins, the recombinant clones were transformed to BL21 (DE3) pLysS cells by heat shock method as described and screened on ampicillin and chloramphenicol plates.

Overnight grown cultures were induced with 0.2mM IPTG at 28°C for 18 hours. The cultures were harvested and using immobilized metal affinity chromatography (IMAC) recombinant proteins were purified from the culture lysate. At each stage, samples were collected for downstream analysis. The procedure was scaled up to get large amounts of recombinant proteins.

Since maximum amount of protein was found in inclusion bodies, the pellet was further processed for denaturation by solubilizing and processed for refolding *in vitro*.

3.2.8 Denaturation and purification of EDIII of KFDV, DENV (1-4) and ZIKV from inclusion bodies

All the recombinant proteins were obtained in inclusion bodies (insoluble form) and thus could not be used for downstream purposes and antibody generation. For solubilization, these inclusion bodies were washed once with **t**riton wash buffer and twice with inclusion bodies wash buffer. Washed pellets were subjected to unfolding or denaturation in TUI 40 buffer.

Denatured protein was further purified using 1ml HisTrap column on ÄKTA^{TM} pure. Triton wash buffer was used for equilibration of column and TUI 750 buffer was used for elution.

For KFDV EDIII, the eluted protein was diluted to achieve final concentration of 3.5M of urea and digested with 1:100 μM ratio of TEV protease for 12 hours. Protein digestion was confirmed by SDS-PAGE and further purified using 1ml HisTrap column.

3.2.9 *In vitro* protein refolding of KFDV EDIII

Purified recombinant KFDV EDIII protein was further employed for renaturation in 250 mL of refolding buffer (50mM Tris-HCl pH 8.8, 0.3 M L-arginine, 1.0M NaCl, 5mM EDTA, 4mM GSH and 4mM GSSG), 20 mg EDIII protein was dialyzed at 4°C by stirring at low speeds for 15-30 days.

3.2.10 Protein concentration estimation and custom made anti-KFDV EDIII polyclonal antibody production

TEV digested recombinant KFDV EDIII was subjected to buffer exchange in 250 ml of T200 buffer containing 2mM β-mercaptoethanol for 1-2 hours at 4°C in a constant slow stirring mode. Protein concentration was estimated using Bradford method [69]. Reference BSA concentration solutions (0.2-2mg/ml) were used for preparing the standard curve.

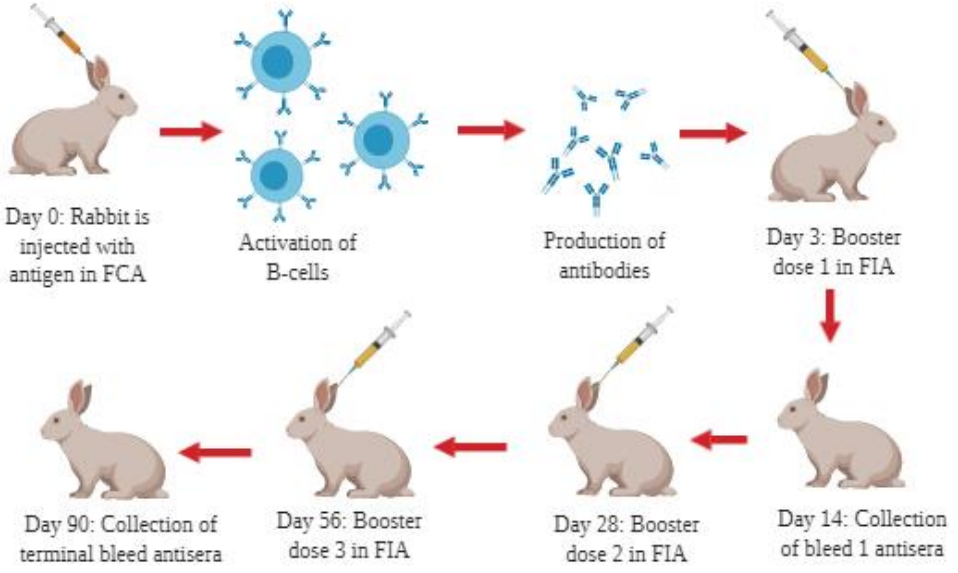


Fig. 3.4 Schematic for generation of pAb in rabbit. The first injection was given by mixing antigen with Freund's complete adjuvant. Subsequently, antisera on day 14 were treated as bleed-1 antisera. On day 3, a booster dose of the antigen was given by mixing antigen with Freund's incomplete adjuvant. On day 90, terminal bleed antisera was collected for downstream analysis.

About 5.0 mg of KFDV-DIII protein was sent to ABGENEX India Private Limited, Infocity, Bhubaneswar for custom pAb production in rabbit. Rabbit sera were collected as bleed-1 sera and terminal bleed sera for downstream analysis.

3.2.11 Size exclusion chromatography

Gel filtration chromatography or size exclusion column chromatography of six denatured EDIII proteins were performed using pre-packed Superdex-200 10/300 column (GE healthcare). For this, firstly the column was equilibrated with TU200 buffer and with a flow rate of 0.5 ml/min, protein samples were injected to column. Heterogeneity in target protein can be easily removed by size exclusion. The column was calibrated with molecular weight standards and the void volume (V_0) was estimated by an inert protein, blue dextran. Molecular weight can be estimated by comparing the elution volume (Ve) of purified protein to that of the elution volume of molecular weight standards and by plotting the standard graph. Thus, the oligomeric nature of protein in solution can also be known by gel filtration method.

Elutes at the peaks were collected and monitored in SDS-PAGE for expected band size of the protein. The obtained elution was used to test the cross-reactivity of rabbit anti-KFDV EDIII antisera against other flaviviruses.

3.2.12 Cross-reactivity experiment (Western blotting)

Anti-KFDV bleed-1 antisera and terminal bleed antisera were tested against KFDV EDIII, DENV serotypes EDIII and ZIKV EDIII. Recombinant EDIII proteins were electrophoretically denatured and transferred onto a PVDF membrane for antibody detection. After transfer at 250 mA at 4°C for 45 min, the PVDF membrane was incubated in blocking solution (5% skimmed milk in TBST) for overnight. Blocking solution was washed multiple times with 1xTBST and the membrane was

incubated in respective rabbit antisera (1:500 ratio dilution of primary antibody) for 12-16 hours. Then primary antibody was washed several times with 1xTBST before incubating in goat anti-rabbit IgG/HRP antibody (1:7000 ration dilution of secondary antibody) for 2 hours. Incubation was carried out at 4°C on a rocking shaker.

Finally, fluorescence was visualized with 1 ml of Serva chemiluminescence reagent for horseradish peroxidase with 0.7 µl of 50% H_2O_2 as substrate. Image Quant LS 4000 (GE Healthcare) was used for subsequent image processing and data interpretation.

Chapter 4

Results and Discussion

4.1 Cloning of KFDV EDIII gene

The KFDV EDIII was synthesized using primer extension and dual asymmetric overlap primers. This was a two-step process in which the CDS for the gene was synthesized by two consecutive PCR reactions. The final result was confirmed by visualizing an expected band size of ~310 bp.

The amplified product was purified, restriction digested and cloned into expression vectors. After transformation to DH5α *E.coli* cells, colonies were screened on ampicillin containing agar plates and also confirmed by colony PCR followed by Sanger sequencing protocol. Colony PCR was carried out using vector-specific and downstream gene-specific primers (universal T7 promoter specific).

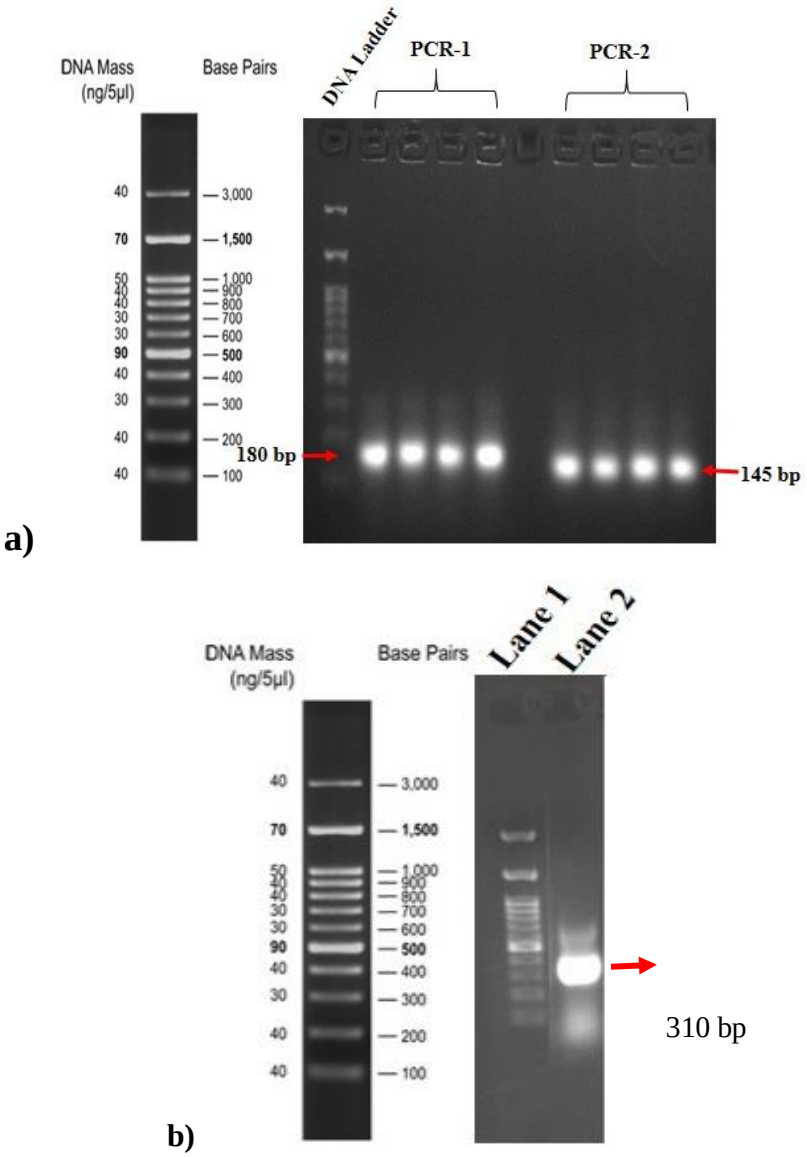


Fig. 4.1 Complete amplification of KFDV CDS *in vitro*. a) Results of initial extension in a two-step PCR resulted in expected band size of 185 bp and 145 bp as described earlier. b) The amplified products from PCR-1 and PCR-2 were extended to the full length of 310 bp as is observed on the gel.

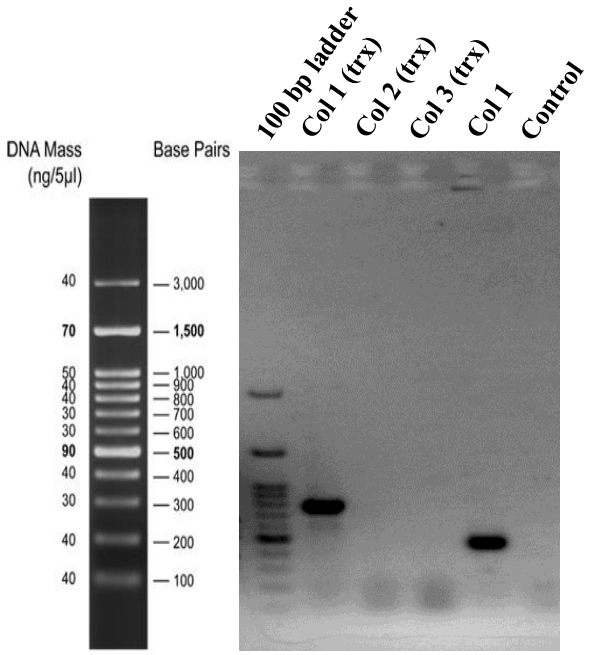
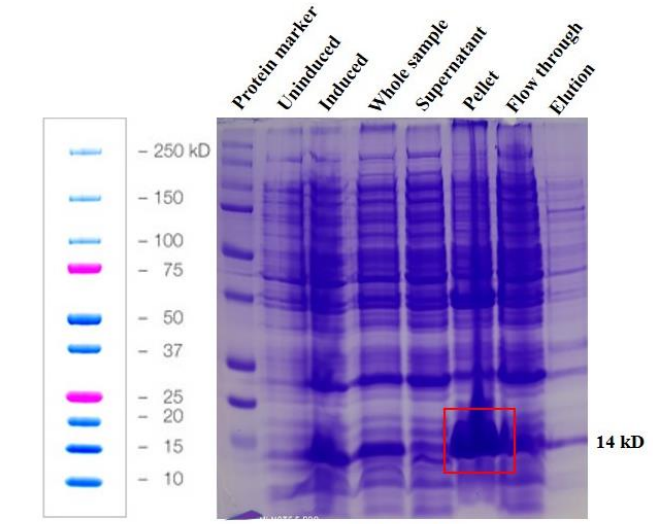


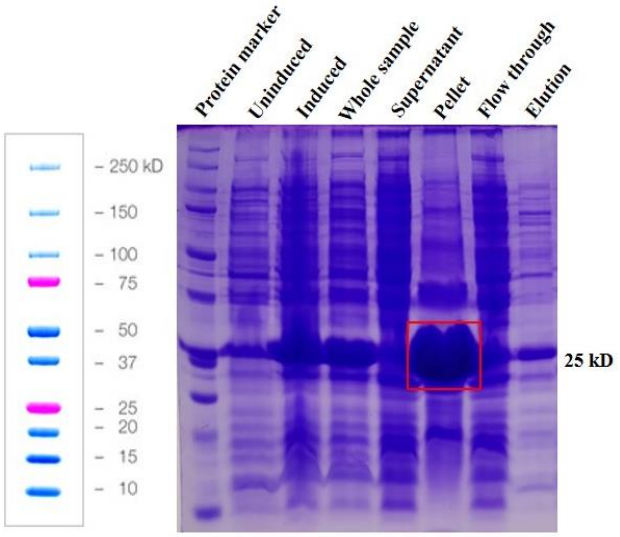
Fig. 4.2 Gel electrophoresis screening by colony PCR. Colonies were PCR screened for a positive band: ~800 bp. Representative picture showing ~800 bp band for pGEX6T1-Trx and ~500 bp for pGEX6T1 plasmid clone. These colonies were further confirmed by analytical restriction analysis and transformed in to expression host.

4.2 Expression and purification of KFDV EDIII gene

The positive clones were purified and transformed in to expression host for protein expression. The overnight grown cultures were induced with 0.2mM IPTG. The protein purified showed bands at expected size from both the recombinant clones: 35.1 at 14kD and 35.2 at 25kD. However, the purified protein was denatured and aggregated into inclusion bodies.



a)



b)

Fig 4.3 SDS- PAGE gel images showing purified recombinant KFDV EDIII. In clone 35.1, N-terminal of KFDV EDIII CDS has Strep-His tagged fusion pepitides whereas in clone 35.2, N-terminal also contains trx tag alongwith Strep-His tag. Though the size of KFDV EDIII is 11 kD, presence of fusion tags add to the molecular weight of purified recombinant protein. Maximum protein was obatined in aggregated form in inclusion bodies.

4.3 KFDV EDIII solubilization and recovery after TEV digestion

Since the maximum amount of protein was obtained in aggregated form, it has to be solubilized for further purification. After several steps of washing with triton wash buffer and inclusion bodies wash buffer, protein was further purified using 1ml HisTrap column on ÄKTA^{TM} pure. Triton wash buffer was used for equilibration of column and TUI 750 buffer was used for gradient elution. EDIII elution was obtained at the elution buffer concentration is ~80% (at concentration of ~0.6M imidazole).

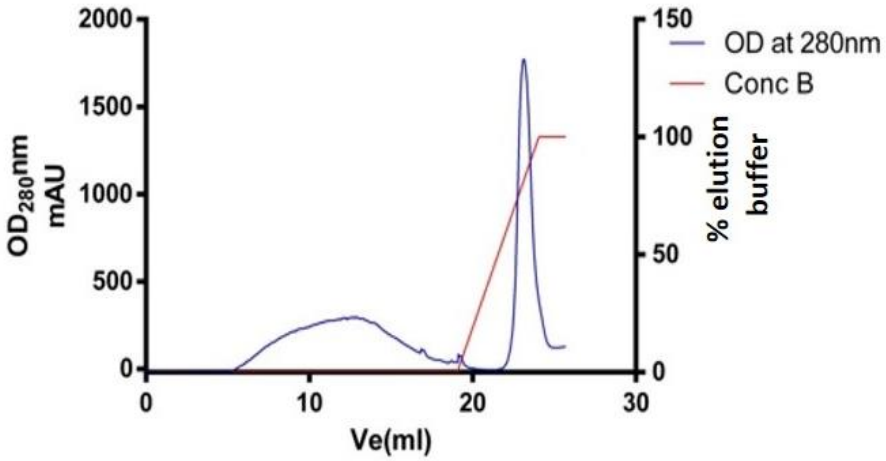


Fig. 4.4 Automated purification of solubilized KFDV EDIII using nickel IMAC on HisTrap^{TM} column. After solubilization and affinity purifcation, 3 ml of protein was eluted at 0.6M imidazole concentration. The quality of automated purified solubilized KFDV EDIII on both the clones was monitored on a SDS-PAGE gel.

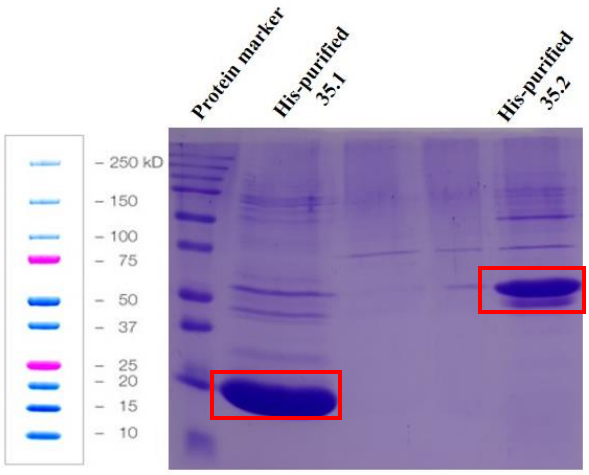


Fig. 4.5 SDS-PAGE of nickel afffinity purified KFDV EDIII protein. His-purified 35.1 shows band size of 11 kD and his-purified 35.2 shows band size of 25 kD.

Since the buffer used for elution conatined 8M urea, the eluted protein needed to be diluted for *in vitro* refolding process. Hence, the eluted protein was diluted to achieve final concentration of 3.5M of urea and digested with 1:100 µM ratio of TEV protease for 12 hours at 21°C. Protein digestion was confirmed by SDS-PAGE.

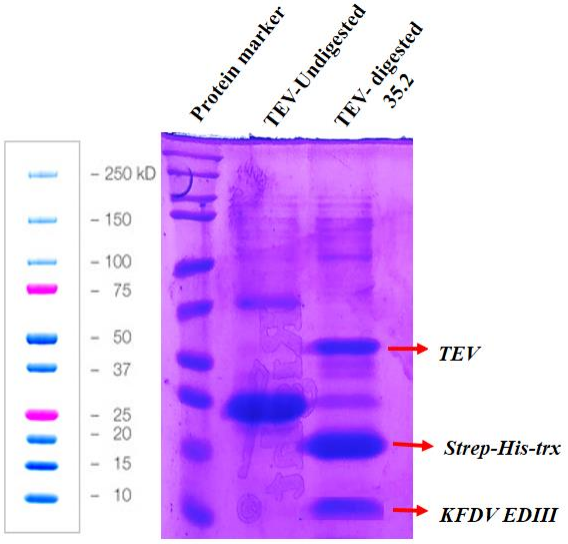


Fig. 4.6 SDS-PAGE of TEV digested EDIII protein of CN 35.2. Lane 1 carries protein ladder. Lane 2 carries undigested clone 35.2 purifed protein.

On digestion with TEV, all three bands are observed at 27 kD (TEV), 15 kD (Strep-His-Trx tagged fusion peptides) and 10.5 kD (KFDV EDIII). TEV digested recombinant KFDV EDIII was subjected to buffer exchange to remove contaminants and to increase yield. For this, the TEV digested protein mix was dialyzed using dialysis tube with 14,000 kD cut-off and incubated in T200 buffer containing 0.2mM β-mercaptoethanol for 2 hours at 4°C.

4.4 KFDV EDIII concentration estimation and generation of pAb

Protein concentration was estimated using Bradford method (69). Standard BSA concentration solutions (0.2mg/mL to 2mg/mL) were used for preparing the standard curve. About 5.0 mg of KFDV-DIII protein was sent to ABGENEX India Private Limited, Infocity, Bhubaneswar for custom pAb production in rabbit.

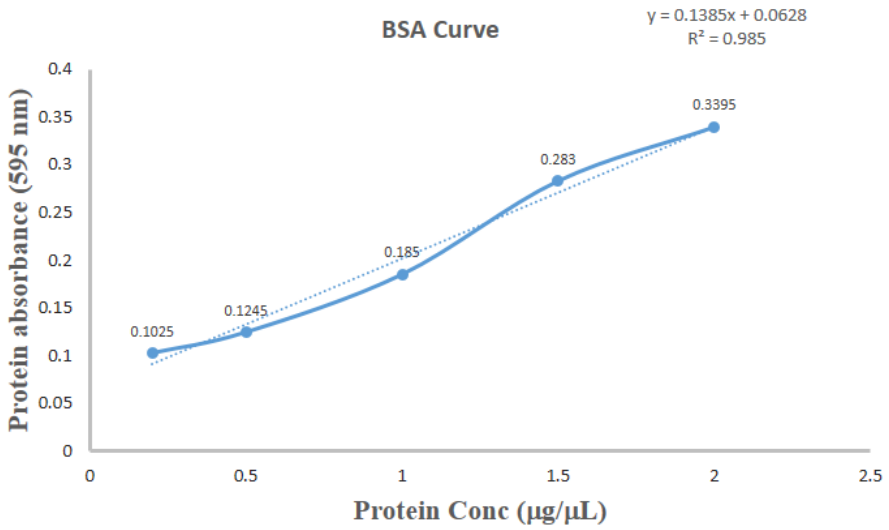


Fig. 4.7 Standard curve for protein quantification us refernce BSA standards. Conc. of KFDV EDIII obtained from the graph was 2.17 µg/µl. About 5.0 mg of KFDV EDIIII antigen was sent for custom made pAb production in rabbit as described earlier.

4.5 DENV (1-4) EDIII and ZIKV EDIII gene amplification and cloning

Gene amplification of EDIII protein of other flaviviruses (DENV1, DENV2, DENV3, DENV4 and ZIKV) was carried out under standard PCR conditions using *Phusion* DNA polymerase.

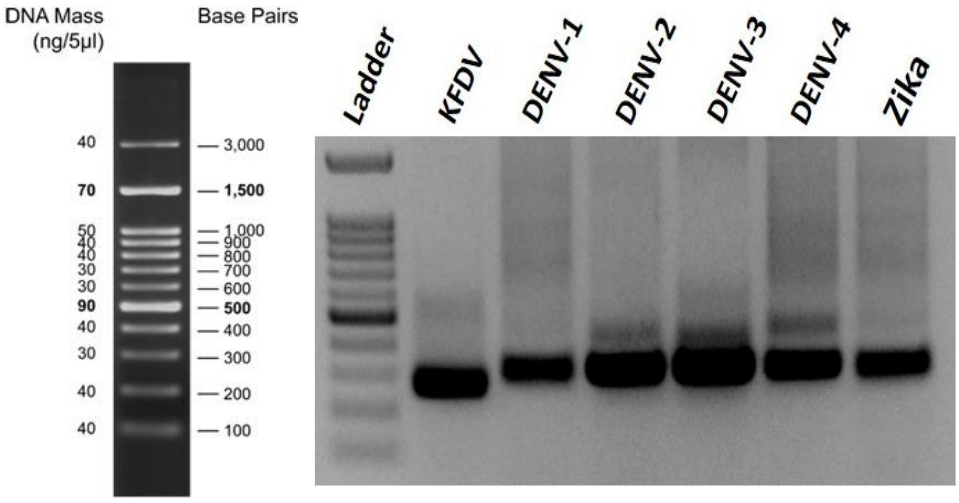


Fig. 4.8 Final amplified gene products of DENV1, DENV2, DENV3, DENV4 and ZIKV (~300 bp each). The maplified products were ran on agarose gel against a DNA ladder. The expected band size was obtained.

Each of the amplified products were purified, restriction digested and cloned into *E. coli* expression vectors. After transformation to DH5α *E.coli* cells, colonies were screened on ampicillin containing agar plates and also confirmed by colony PCR using vector and gene-specific primers (universal T7 promoter specific). Clones were further confirmed by Sanger sequencing.

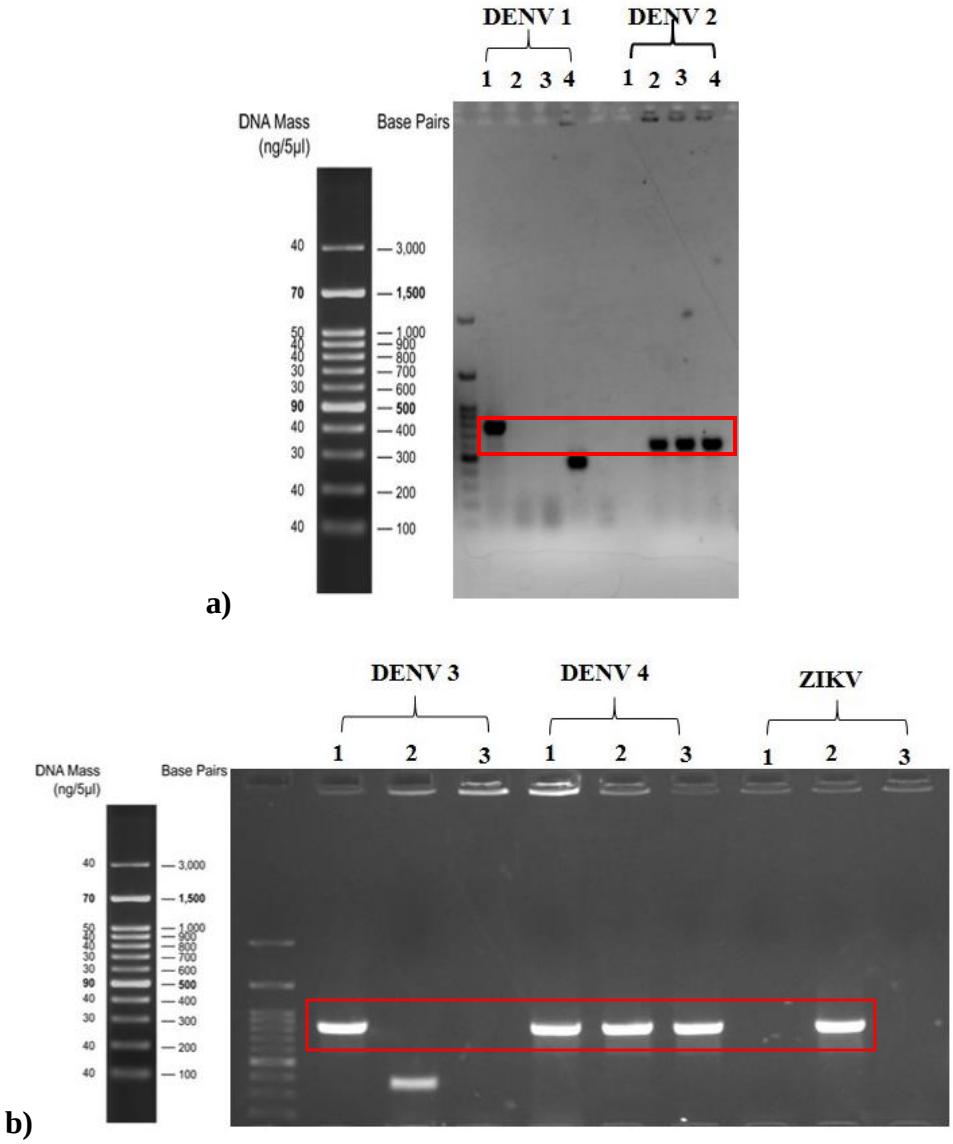
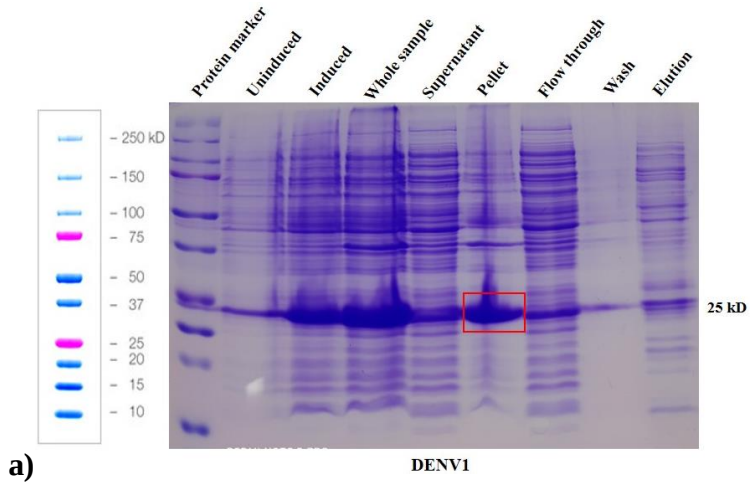


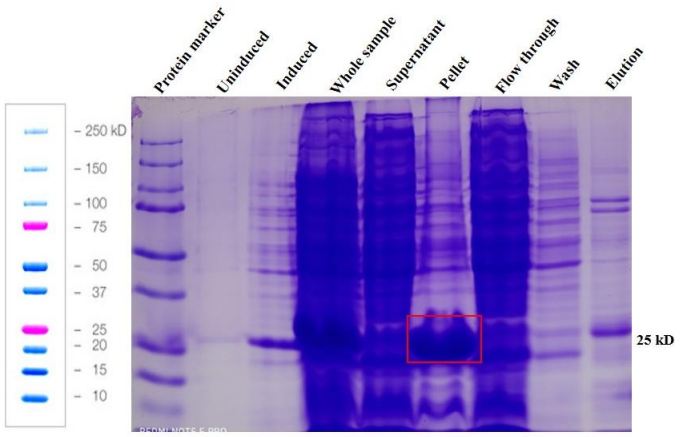
Fig. 4.9 Colony PCR for EDIII a) shows single positive colony for DENV1 and 3 colonies for DENV2 and b) shows 1 positive colony for DENV3, 3 colonies for DENV4 and 1 colony for ZIKV. The clones from positive colonies were grown overnight at 37°C at 220 rpm. Recombinant clones were purified and transformed into expression host.

4.6 Expression and purification of recombinant DENV and ZIKV EDIII proteins

Recombinant clones were transformed and induced to express respective EDIII proteins. The purified protein showed bands at expected size. However, the maximum amount of purified protein was denatured and aggregated into inclusion bodies in all the cases. So, protein was further purified from the aggregated bodies as described earlier for KFDV EDIII.



a) DENV1



b) DENV2

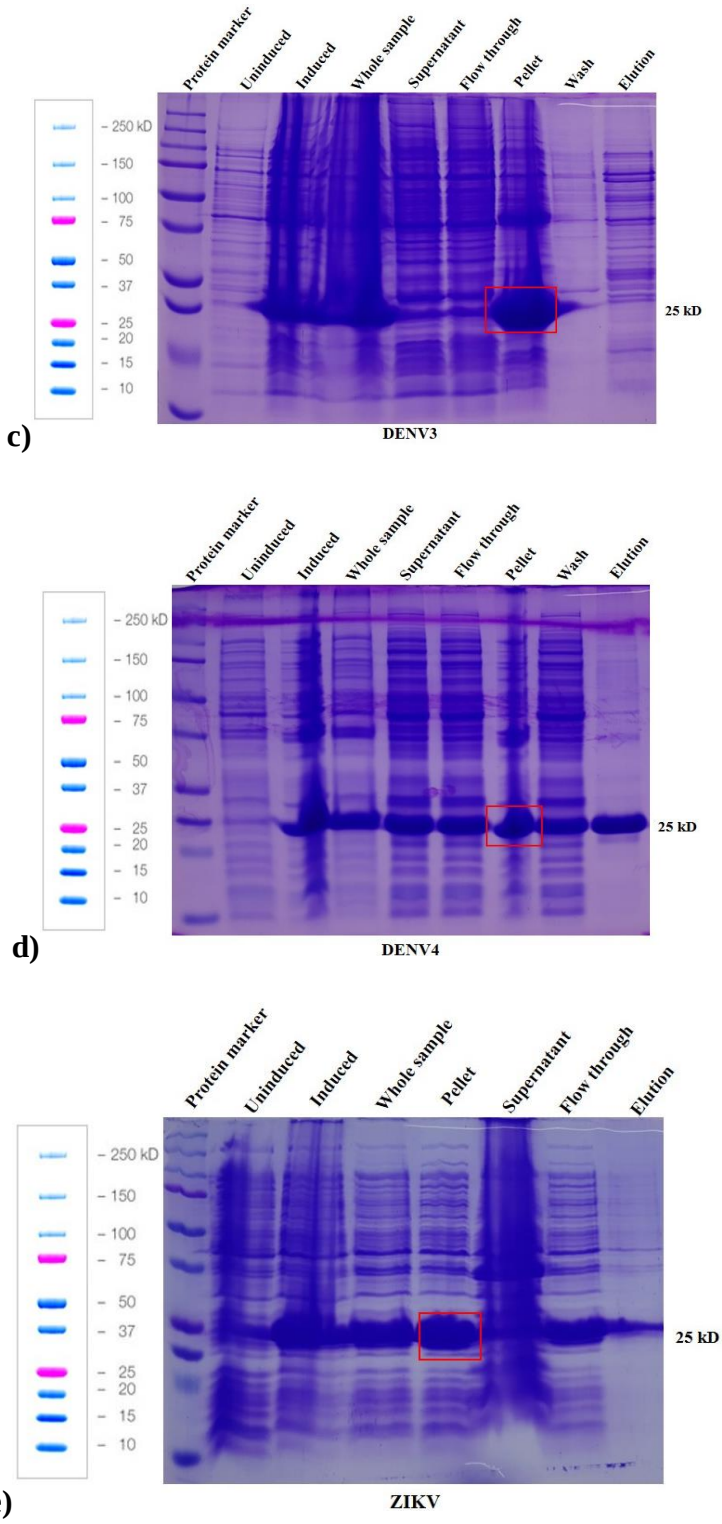


Fig. 4.10 SDS- PAGE gel images showing expression of different EDIII proteins [(a) to (e)]. In all cases, maximum amount of protein is found in aggregated form in inclusion bodies.

The aggregated inclusion bodies were washed with triton wash buffer and inclusion body wash buffer, and further denatured in the presence of high concentrations of urea (8M) to make them soluble.

4.7 Size exclusion chromatography

Size exclusion chromatography separates the molecules on the basis of size in the solution. As the sample passes through the column, molecules which are too large to penetrate the pores of the packing are excluded and remain in the interstitial volume (V_o). These molecules are the first to be eluted. The curve is constructed by measuring the peak elution volumes (V_e) of a series of monodisperse polymer standards with known peak molecular masses. The elution volumes decrease with increase in the logarithm of molecular mass (70).

EDIII proteins of the flaviviruses were purified using gel filtration method to obtain separated elution peaks as shown in the graph (Fig 4.12).

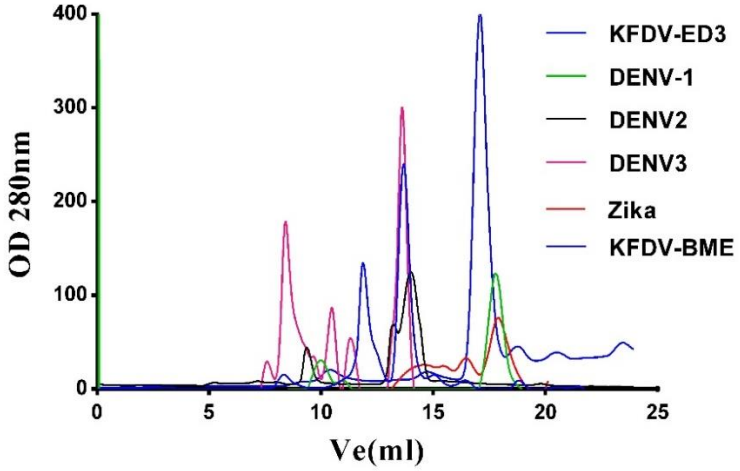


Fig. 4.11 Size exclusion chromatogram. Mono-disperse peaks of the eluted proteins were obtained at different peaks as shown. Peaks were collected and monitored in SDS-PAGE. The elution profile also shows the shift in profile of KFDV EDIII after adding 14.4 mM β-mercaptoethanol (βME). This suggests that the KFDV EDIII was being eluted close to its

structure in dimeric state. On addition of β-mercaptoethanol, peak was shifted to lower elution volume due to reduction from dimeric to monomeric state. The peaks obtained at different elution volumes were collected monitored on SDS-PAGE.

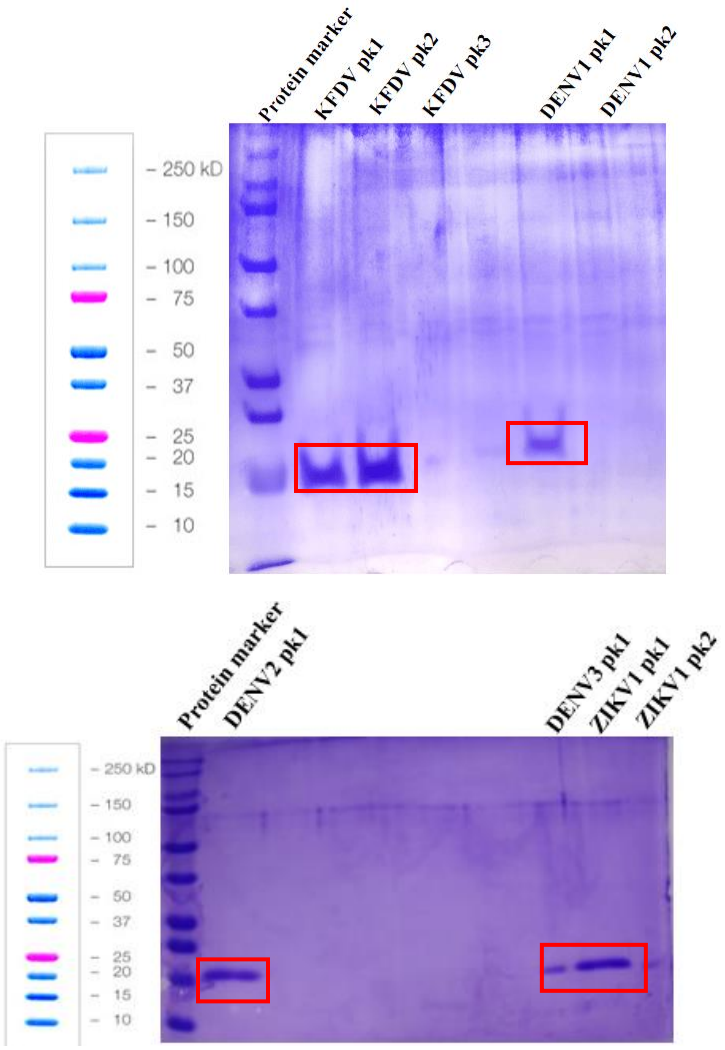


Fig. 4.12 Size exclusion peak of elutions. The peak elutions obtained after gel filtration of the EDIII proteins were monitored over the SDS-PAGE which showed the bands at ~10kD for each protein. The desired solution peaks were further used to test cross-reactivity.

4.8 Antibody Cross-reactivity

Due to sequence similarity of KFDV with that of mosquito borne flaviviruses (between 30-35%) and with tick-borne flaviviruses (between 60-85%), antibodies generated against KFDV was proposed to cross-react with other flaviviral EDIII proteins.

Currently, KFDV is diagnosed by IgM based ELISA kit which is unable to discriminate between KFDV and its alike diseases. To address this issue, we generated custom-made polyclonal antibodies in rabbit against KFDV EDIII. First bleed antibodies (after 2nd injection on 14^{th} day) and terminal bleed antibodies (at the end of 3 months on 90^{th} day) were collected.

We performed Western blot using first and terminal bleed antisera as primary antibodies and anti-rabbit HRP conjugated goat antibodies as secondary antibodies. Interestingly, we found that the first bled antibodies showed good interaction with KFDV EDIII along with that of DENV1, DENV3 and ZIKV EDIII proteins. Whereas the terminal bleed interacted only with KFDV EDIII antigen. This suggests that constant antigen dose to the animal would have resulted in increase the specificity antibodies towards the antigen. This might have resulted in clonal expansion of exact B cell receptor bearing population with repeated exposure to antigen.

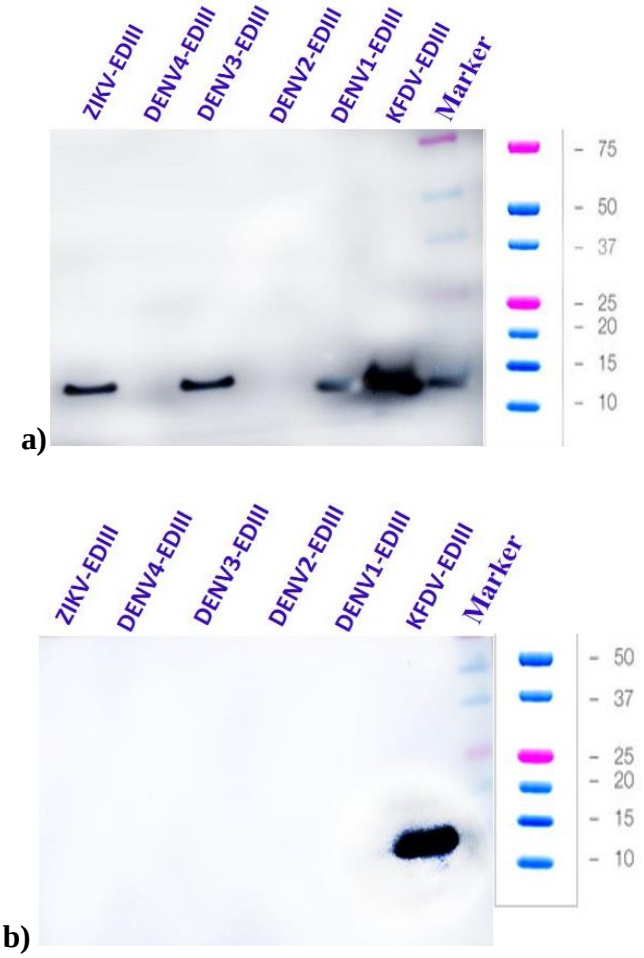


Fig. 4.13 Western blot result showing anti-KFDV EDII antibody cross-reactivity. 0.5 mg of purified antigen was loaded to each well of the gel as depicted in the figure. After the protein was transferred to PVDF membrane, rabbit antisera was probed to obtain Western blot result as described earlier a) First bled antisera showing reactivity to five flaviviral EDIII antigen and b) terminal bleed antisera against the said antigens of five different flaviviruses

Chapter 5

Conclusions and Future Prospects

Due to the sequence and structural similarity of flavivirus envelope protein (E), antibodies against one virus could cross react with other flavivirus antigens. This unwanted situation interferes with diagnosis and host pathogenesis. For an example, as anti-DENVI antibodies could very well cross react with other DENV serotypes (e.g. DENVII). In the human host, exposure to one DENV serotype could produce antibodies, which in future could interact with other serotypes. In fact, this mechanism exacerbates the pathogenesis which enhances antibody-medicated internalization of infectious virion to the antigen present cells. Where the internalized virion could replicate quickly and worsen the disease condition. This peculiar phenomenon is well observed in flaviviruses where cross reactivity of DENV with that of ZIKV etc. is reported. Alternatively, the same principle interferes with accurate viral diagnosis. For this reason, differential diagnosis become challenging. In this project, we first employed bioinformatics approach to identify signature sequences of KFDV. Further, wet laboratory experiments were performed which was based on the result of previous stage. Signature sequences were identified and custom antibody against KFDV EDIII was generated. Additionally, we tested the cross reactivity of anti-KFDV EDIII antibodies with that of other related flaviviruses (eg. DENV). In this project we here report that

- For the first time we created model structure of KFDV E protein using computational biology approach.
- We show that other flavivirus-specific antibodies (mAb 19/1786) could bind to the KFDV E protein
- We successfully cloned and expressed of KFDV EDIII protein.

- Custom polyclonal anti-KFDV EDIII antibody were generated successfully.
- We report that the first and terminal bleed anti-KFDV EDIII antibodies showed differential cross-reactivity phenomena to other flaviviral antigen.
- However, the terminal bleed was specific to KFDV EDIII.
- We therefore propose that our antibody could be useful in direct detection of KFDV antigens in human host
- I look forward to seeing our antibody being turned to a test kit in near future
- Based on this result we propose that appropriate humanized therapeutic antibody development has good possibility
- Further this antibody would help in resolving crystal structure of KFDV E protein
- Whole virus structure could be resolved using this antibody

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