Peptides Containing T Cell Epitopes of ZIKAVirus Envelope Protein Sharing Homology To Other *Flavivirus* Species

A Dissertation

Submitted in the partial fulfillment of the requirement for

the award of the degree of

Master of Science

In

Biotechnology

Submitted by:

VanitaKinra
301601023

Under the guidance of:

Dr. Manoj Baranwal
(Associate Professor)

Department of Biotechnology
Thapar Institute of Engineering&Technology
Patiala, Punjab.

CERTIFICATE

This is to certify that the project entitled "**Peptides containing T cell epitopes of ZIKA virus Envelope protein sharing homology to other Flaviviruses species**" submitted by VanitaKinra in the partial fulfilment of the requirement for the award of degree of **Master of Science** in Biotechnology to Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is a record of student's own work carried by her. The report has not been submitted for the award of any degree or certificate in this or any other University or Institute.

Dr. Manoj Baranwal
Associate Professor, Supervisor
Department of biotechnology,
TIET,Patiala

DECLARATION

I hereby declare that the work which is being presented in dissertation entitled "**Peptides containing T cell epitopes of ZIKA virus Envelope protein sharing homology to other Flaviviruses species**"submitted by me for the award of the degree of **Master of Science** in Biotechnology to Department of Biotechnology, Thapar Institute of Engineering & Technology, Patialais an authentic record of my work during the period from Jan 2018 to June 2018, under the supervision of **Dr. Manoj Baranwal**, Associate Professor, Thapar Institute of Engineering & Technology, Patiala. Further, I declare that no part of this dissertation has been submitted to any other University/Institute for the award of any degree in India or abroad.

Place: Patiala

Date: 21.8.2018

Vanita

VanitaKinra

ACKNOWLEDGEMENT

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely fortunate to have got this all along the completion of my research work. Whatever I have done is only due to such guidance and assistance and I would not forget to thanks them.

I am highly obligated to my guide **Dr. Manoj Baranwal** for their guidance and constant supervision as well as for providing necessary information regarding the project & also for their support in completing the project.

I express my thanks to **Dr. Moushumi Ghosh**, Professor and Head, Departmennt of Biotechnology, Thapar Institue of Engineering Technology, Patiala, for providing best laboratory facilities.

I would like to express my gratitude towards all the research scholars **Mr. Sahil Jain** and **Ms. Neha Srivastava** for their kind co-operation and encouragement which help me in the completion of this project. I would also like to express my special thanks to my lab mates **Ms. Garima Batish** and **Ms. Tania Patwal** for giving me thoughtful advices and motivation to do my work with precision.

My thanks and appreciations also go to my colleague **Ms.Garima Sharma, Ms. Malvika Mehta, Ms. Shreya Arora** whose joyful company kept me alive throughout out my entire stay at Thapar. They willingly helped me out with their abilities in each situation.

I would like to mention the unconditional support from my family, whose blessings boosted me to achieve my destination. My loving thanks to my father **Mr.Vasdev Kinra**, my mother **Mrs. Bhawna Kinra**, my elder sisters **Mrs. Hema Kinra** and **Ms. Manisha Kinra** and my brother in law **Mr. Rahul Mutneja**.

Place: Patiala

Date: 21. 8- 2018

VanitaKinra

TABLE OF CONTENTS

ABSTRACT

LIST OF TABLES

LIST OF FIGURES

Chapter 1 INTRODUCTION	**4**
Chapter 2 REVIEW OF LITERATURE	**6**
2.1 Zika Virus: History	6
2.1.1 First Isolation	6
2.1.2 First evidences of human infection	6
2.1.3 Spread of Zika (1951–present)	6
2.2 Zika Virus: Taxonomy	7
2.3 Flaviviridae: life cycle	7
2.4 Flaviviridae viruses: Structure	8
2.5 Flaviviridae Proteins	10
2.5.1 Structural Proteins	10
2.5.2 Non Structural Proteins	11
2.6 Envelope protein of various flaviviridae members	12
2.6.1Zika Virus envelope protein	12
2.6.2 Dengue Virus envelope protein	13
2.6.3 West Nile Virus envelope protein	14
2.6.4 Japanese encephalitis virus envelope protein	15
2.6.5 Spondweni virus envelope protein	16
2.7 Comparison of e-protein of various flaviviridae members	16
2.8 Flaviviridae viruses: diseases and symptoms	18

2.9 Flaviviridae diseases: treatment and prevention 20

2.10 Vaccines against flaviviridae 20

2.10.1 Vaccines against Zika virus 20

2.10.2 Vaccines against Dengue virus 21

2.10.3 Vaccines against West Nile Virus (WNV) 22

2.10.4 Vaccines against Japanese encephalitis virus (JEV) 22

2.11 Peptide Vaccines 22

2.12 Immunoinformatics 24

Chapter 3: MATERIAL AND METHODS 25

3.1 Sequence Retrieval 25

3. 2 Multiple Sequence Alignment 25

3.3 Conservancy Analysis 25

3.4 Peptide Screening 26

3.4.1 Peptide Match 26

3.4.2 AlgPred 26

3.4.3 ToxinPred 26

3.5 Mapping of peptide fragments 27

3.6 Cabs dock analysis 28

Chapter 4 RESULTS AND DISCUSSION 29

4.1 Peptide selected for this study 29

4.2 Sequence Retrieval 29

4.3 Sequence Comparison 29

4.4 Peptide identity analysis 33

4.4.1 Percentage idenhtity to previously identified immunogenic Zika peptides 34

4.5 Peptide Screening 36

4.6 Mapping of peptide fragments 36

4.7 Zika HLA-peptide interaction analysis: CABSdock 39

Chapter 5 CONCLUSION 41

REFERENCE 42

ABSTRACT

The concept of peptide-based vaccines against viral infection has made significant progress and it has been reached to different clinical trials. Envelope protein in flaviviruses virus play an important role in assembly of virions, host cell binding and membrane fusion and cover majority of the virion surface, which makes them ideal candidate for peptide based vaccine development In the present study, conserved peptides containing multiple T cell epitopes of Zika virus envelope protein were compared from flaviviruses envelop protein for sequence homology todevelop a cross protective vaccine effective against various flaviviridae members. Seven peptides containing CD4+ and CD8+ T cell epitopes were taken from previous report. These peptides were looked for their homology in four flavivirus (Dengue, West Nile Virus, Japanese Encephalitis virus, and Spondweni virus).Four peptides (P2, P5, P6 and P7) containing multiple epitopes of Zika virus Envelope protein were observed to have a remarkable sequence identity (≥ 70%) in all four flaviviruses. Screening studies reveal thatall the peptides belonging to different flaviviruses having sequence identitywere non-allergen and non self.Peptides have shown RMSD value (CABS-dock) less than three which indicates a strong binding potential with HLA molecules.Hence, it is suggested that these peptides may be considered for synthetic peptide vaccine design against Flaviviruses.

LIST OF TABLES

TABLE	*PAGE NO.*
Table 1: Pairwise sequence comparison of the E proteins from different Flaviviruses	**18**
Table 2: Disease and symptoms of flaviviruses	**18**
Table 3: Host, vector and incubation time of flaviviruses	**19**
Table 4: Human ZIKV vaccines which are currently in clinical development	**21**
Table 5: Vaccines against dengue virus that are licensed or in different clinical stage	**21**
Table 6: Human West Nile Virus vaccines which are currently in clinical development	**22**
Table 7: Human JEV vaccines which are currently in clinical development	**23**
Table 8: PDB id's used to locate identified peptides	**28**
Table 9: PDB ids with corresponding HLA allele and resolution	**29**
Table10: Selected peptides containing multiple T cell epitopes of Zika virus envelope protein	**30**
Table 11: Total number of sequences available in the database and the number of unique sequences downloaded	**31**
Table 12: Comparison of peptides showing the best percentage identity	**34**
Table 13: Peptides in different flaviviruses	**35**

LIST OF FIGURES

TITLE	*PAGE NO.*
Figure 1: Classification of Zika virus	**7**
Figure 2: Flavivirus lifecycle	8
Figure 3: Flavivirus structure	9
Figure 4: Flavivirus genome organization	12
Figure 5: Envelope protein structure of Zika virus	13
Figure 6: Dengue virus Envelope protein structure	14
Figure 7: Envelope protein structure of West Nile Virus	15
Figure 8: Envelope protein structure of JEV	16
Figure 9: E protein comparison of flaviviruses	17
Figure 10: Mapping of peptides on 3 D structure of Envelope protein	37
Figure 11: Mapping of peptides on 3D structure of Envelope protein of Dengue virus serotypes	38
Figure 12:RMSD values for native and peptides (P2, P5, P6 and P7) of Zika Envelop protein	39
Figure 13: Best docking poses obtained via CABSdock.	40

1. INTRODUCTION

Zika virus (ZIKV) infection is a mild febrile disease which is communicable to humans throughan infected *Aedes* mosquito (Sikka *et al.*,2016). ZIKV is an emerging arbovirus infection endemic in multiple countries spread from Asia, Africa and Oceania with intervallic cases in America and Europe (Plourde *et al.*,2016). ZIKV infection causes a characteristic rash, fever, headache, conjunctivitis and neurological disorders such as microcephaly in infected mothers to newly born babies.

Zika virus belongs to a member of viruses called flaviviruses, which includes West Nile virus (WNV), Japanese encephalitis virus (JEV), dengue virus (DENV) and Spondweni virus(SPOV) (Lanciotti *et al.*, 2007).The flavivirus virions surrounded by a lipid bi-layer which comes from the host cell. The virions contain capsid, viral RNA and an outer glycoprotein shell. When virus enters in human body, envelope (E) protein of the virus forming the glycoprotein shell help in internalization of the virus via clathrin-mediated endocytosis by binding to cell surface receptors (Gollins and Porterfield, 1984; Ishak *et al.*, 1988; Van der Schaar *et al.*, 2007).The 3' and 5' ends of the flaviviridae genome contain 100 and 400-700 nucleotide bases in non-coding (NC) regions. A translation of open reading frame (ORF) result in a formation of single polyprotein which finally culminates into seven non-structural (NS) proteins (NS1,NS2a,NS2b,NS3,NS4a,NS4b and NS5) and structural proteins(C, E and M). Notably, these ten proteins are common to all flaviviridae members. Envelope protein (E) covers majority of the virion surface and consists of three structural domains (DI,DII and DIII) (Goo *et al.*, 2017).E protein present on the surface of ZIKA virus has a major impact in the binding of receptor and fusion of membrane. The third domain of E protein includes a number of epitopes which are targets in neutralizing antibodies, serological tests and vaccines .The non-structural proteins plays a main role in replication of genome, processing of polyprotein, and structural proteins helps in the formation the virus particle. Virus enters via cellular receptors of skin cells, and migrates to the lymph node and the blood stream. Hamel *et al.*, (2015) concluded that ZIKV might enter through human skin cells that involve keratinocytes, fibroblasts and immature dendritic cellsas permissive to the virus isolate that caused the epidemic in French Polynesia. The phosphatidyl serine receptor AXL (adhesion factors) which facilitate infection was identified as performing a most important role inflavivirus entry into cells, while cellular autophagy enhanced viral replication.

The sequence identity within DENV serotypes was analyzed that 98.1, 98.6, 98.9 and 99.0% for serotypes one to four respectively. On the basis of polyprotein, among all Flavivirus members, sequence identity (55.1-56.3%.) of DENV and ZIKV is highest WNV, JEV and DENV1-4. The maximum sequence identity in DENV1-4, WNV and JEV is from 54.0 to 57.8%. The higher identity of E protein is among DENV1 and DENV3 is 57.8%

Vaccine development against flaviviruses remained a challenging to the scientific community. Scientists at NIAID's Vaccine Research Centre (VRC) have developed manytype of vaccines such as DNA-based vaccines, live attenuated and inactivated vaccines of flaviviruses which are under clinical trials. One of the recent advancement has been done in the direction of peptide based vaccine. Traditional approach in identification of peptide eliciting immune response is is a time-consuming, expensive and complex process. Application of immune informatics reduces the time frame in finding the peptide containing multiple epitope. In the present study, seven identified peptides containing multiple epitopes of Zika envelop protein from previous report were looked for their sequence homology in other flaviviruses. Further, molecular docking were carried out to assess the binding strength will different HLA molecules. The present study may help in finding immunogenic candidates as a cross protective vaccine target against different flaviviruses.

Hence the objective of the present study are:

1. To find the peptides containing multiple epitopes of ZIKA virus envelope protein showing homology to other flaviviruses

2. Screening of identified peptides for non-desirable response

3. Molecular docking of peptides with HLA molecules

2. REVIEW OF LITERATURE

2.1 Zika virus: History

2.1.1 First isolation

In April 1947, the first case of Zika virus was reported in the Zika forest of Uganda (near Lake Victoria) (Sikka*et al*., 2016; Dick *et al*., 1952). The source of this virus was a rhesus macaque monkey. In Jan 1948, the virus was again isolated in the same region from the mosquito *Aedes africanus.*

2.1.2 First evidences of human infection

Zika was found to infect humansin 1952 on analyzing the outcomes of a serological survey in Uganda (Sikka*et al*., 2016). Human sera (6.1%) tested positive for neutralizing antibodies. An investigation of the jaundice outbreak in 1954 sheds light on the presence of a virus in a patient, later reported to be Spondweni virus (SPOV), a virus closely related to Zika. In 1956, an incidence of self-inflicted SPOV infection was reported.

2.1.3 Spread of Zika(1951 to till date)

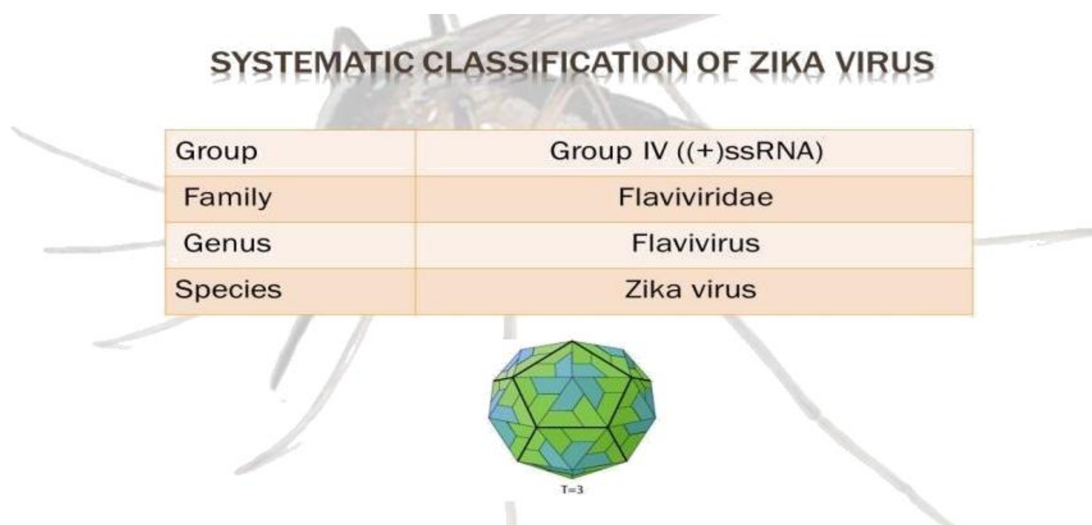
Cases of human infection in African and Asian countries were reported in serological surveys during the 1960's. The first case of human infection was reported by Simpson in 1964. Unfortunately, the researcher himself got infected during the study.

An outbreak of Zika virus disease (initially thought to be dengue or chikungunya disease) was reported on the island of Yap in the Federated States of Micronesia in April 2007 (Duffy *et al*., 2009). Symptoms of the disease included rash, arthralgia and conjunctivitis.

Zika virus came into limelight in year 2015-2016 due to its epidemic spread in American continents with extensions to Europe. In January 2016, Zika infection was pronounced as emergency concerned to public health by the WHO (World health organization) (Roberts *et al*., 2016). In February 2016, many cases of microcephaly and Guillain-Barre syndrome were reported in Brazil, which is said to be associated with Zika virus outbreak by the WHO. The organization also warned of a scenario where the virus shall be present throughout the American continent by the end of the year.

2.2 Zika virus: Taxonomy

Zika virus is an arbo virus which is an arthropod-borne virus classified into the flaviviridae family and the genus flavivirus. Zika is primarily spread to people through the bite of an infected daytime-dynamic female mosquitoes (*Aedesaegypti and AedesAlbopictus*) (Abushouk and negida, 2016). In the late 1800s or early 1900s, phylogenetic analyses shows that ZIKV has two distinct lineages one includes the African strains, and the other the Asian and American strains. Many studies showed that, from South America an Asian lineage is isolated which replicate at low levels in tissue months after the initial infection.


SYSTEMATIC CLASSIFICATION OF ZIKA VIRUS

Group	Group IV ((+)ssRNA)
Family	Flaviviridae
Genus	Flavivirus
Species	Zika virus

Figure 1: Classification of Zika virus (slideplayer.com)

2.3 Flaviviridae: Life cycle

The Flavivirus virion surrounded by a lipid bi-layer which comes from the host cellthat surrounds the capsid and viral RNA and consist of an outer glycoprotein shell. During virus entry, envelope (E) proteins of the virus forming the glycoprotein shell help in internalization of the virus via clathrin-mediated endocytosis by binding to cell surface receptors (Ishak *et al*., 1988; Van der Schaar *et al*., 2007; Gollins and Porterfield, 1984) (Figure 2). The viral glycoprotein structure reorganizes owing to the low endosome pH, which leads to viral membrane fusion and endocytic membranes fusion resulting in the liberation of viral RNA in the cytoplasm (Modis *et al*., 2004; Bressanelli *et al*., 2004). Translation of the released RNA results in a single polyprotein. The latter is processed by viral and cellular proteases into structural and non-structural proteins .Cytoplasm of host

cells is the site of replication and the non-structural proteins plays a major role in actively replicating the viral RNA (Mackenzie *et al*., 2001).

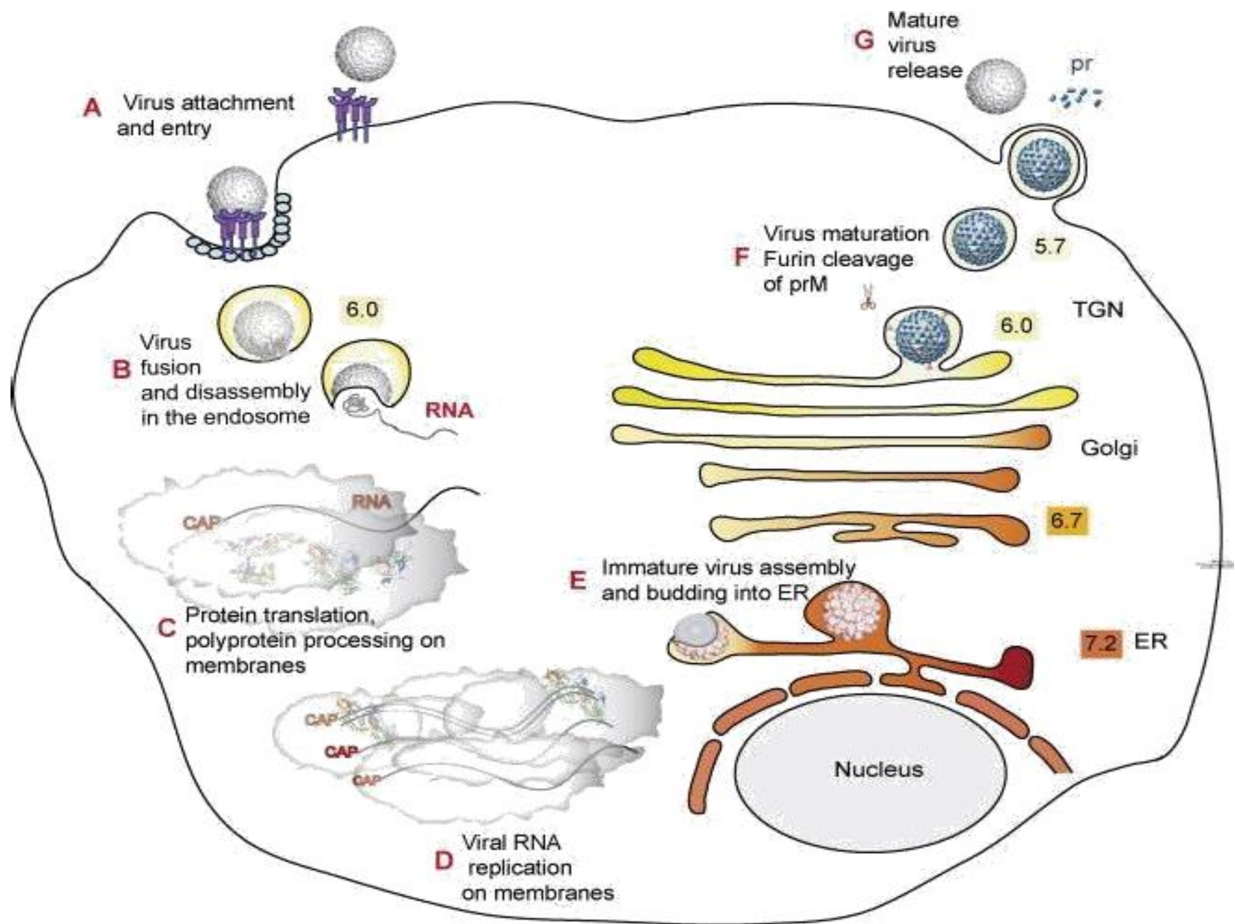


Figure 2: Flavivirus lifecycle (Perera et al., 2008)

Immature and non-infectious viruses containing the M protein precursor membrane are formed in endoplasmic reticulum before the release of mature and infectious virus particles that take place through furin cleavage of precursor membrane in the trans-Golgi network (Stadler*et al*., 1997; Yu *et al*., 2008). This cleavage also enables the E protein to undergo conformational changes which is required for virus entry v.i.z., receptor-binding and membrane fusion (Kaufmann *et al*., 2011;Stiasny and Heinz, 2006).

2.4 Flaviviridae viruses: Structure

The flaviviridae are spherical viruses with a diameter of 30-50 nanometres. They consist of a single positive-strand RNA genome with 11-12 kb in length, with a 5' cap and devoid of a poly A tail. The genome is enclosed by an icosahedral nucleocapsid, 25-30 nm in

diameter (LR and JT, 2001). The latter is surrounded byan envelope lipid bi-layered with small spike like surface projections. This envelope consists of E and M proteins. E protein is glycosylated and is the major antigenic determinant on virus particles while the M protein, which acts as a stabilizing factor and protects the E protein (Sanchez *et al*., 2005).

The 3' and 5' ends of the flaviviridae genome contain non-coding (NC) regions. The length of these NC regions was 100 and 400-700 nucleotide bases respectively (Williams *et al*., 2007). A single open reading frame (ORF) translation of the genome result in a single polyprotein which finally culminates into sevennon-structural (NS) and three structural proteins (C, M and E). The sevennon-structural (NS) proteins were designated as NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. Notably, these ten proteins are common to all flaviviridae members.

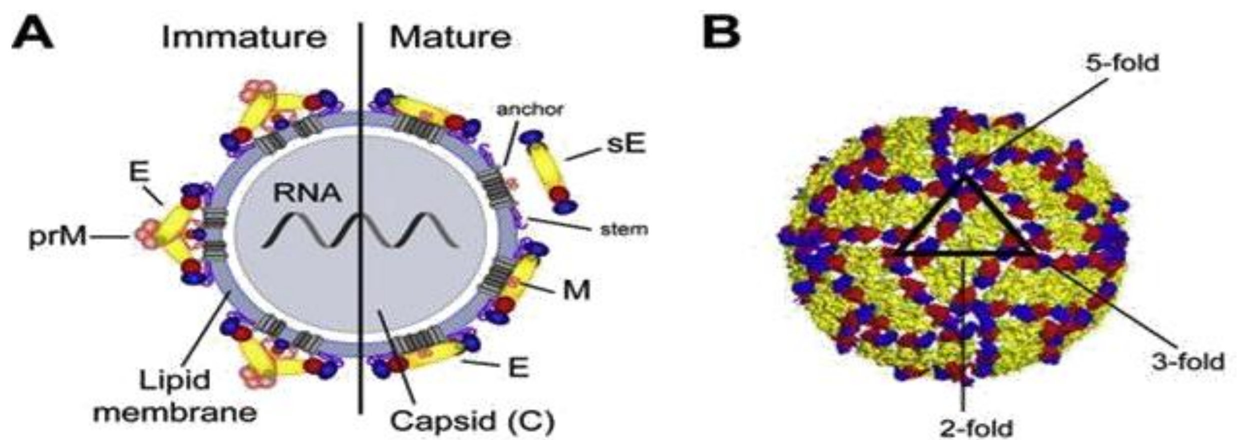


Figure 3: Flavivirus structure (Heinz and Stiasny, 2012)

2.5Flaviviridae proteins

2.5.1 Structural proteins

Three structural proteins viz., core or capsid (C), membrane (M) and envelope (E) proteins are encoded by genes in the same order present downstream of the 5' NC region of the genome.

Capsid protein (C)

The core protein is composed of approximately 120 amino acids. It weighs nearly 11 kilodaltons (KD) and has a hydrophobic central region. It plays a significant role in icosahedral nucleocapsid formation. It also forms a complex with the viral RNA by binding to it with the help of basic residues located on its N and C terminals. Moreover, capsid proteins pave the way for viral assembly by interacting with the host cellular membrane (Markoff *et al*., 1997).

Membrane protein (M)

The M protein is composed of approximately 165 amino acids and is present only in mature virions. The gene encoding for this protein is firstly translated into a 26 KD immature form called prM (Lindenbach and Rice, 2003). The latter guards the E protein against degradation during viral assembly (Bray and Lai, 1991). The C terminal of prM comprises of the M protein. prM is cleaved with the help of furin during the virion exit from the cell resulting intoa 8 KD mature M protein and a “pr” segment (Stadler *et al*., 1997).

Envelope protein (E)

The E protein is composed of approximately 495 amino acids and weighs around 50 KD, making it the largest of the three structural proteins. It is a glycosylated type I membrane protein consists of cellular receptor-binding site(s) (Lorenz *et al*., 2002). This protein is part of the outer surface of lipid bi-layered envelope and is the prime target for neutralizing antibodies. It playsa crucial role in viral entry and membrane fusionand thus a prime target for therapeutic and vaccine developments. (Sanchez *et al*., 2005; Stiasny *et al*., 2007).

prM-E heterodimers exist as spike like trimers on the surface of immature virions where the presence of 90 E homodimer in herringbone-like arrangement is observed on mature virions(Zhang *et al*., 2003; Kuhn *et al*., 2002 and Mukhopadhyay *et al*., 2003).

2.5.2 Non-structural proteins

NS1

NS1 protein, (48 kilodaltons) may exist in moreover cell-surface, cell-associated, or extracellular non-virion forms (Macdonald *et al*., 2005). The latter associates with cell surfaces and corresponds to a robust humoral immune response. It also plays a very significant role in viral RNA replication, immune evasion and virus morphogenesis.

NS2

The coding region of NS2 codes for proteins NS2a and NS2b.The NS2a play a vital role and help in regulation of the viral RNA replication complex and in virion assembly. NS2b is a small protein (~14kDa) that acts as a cofactor for the viral trypsin-like serine protease function of NS3, and also act as a helicase (Bollati *et al*.; 2010).

NS3

NS3 has serine protease, RNA helicase and nucleotide triphosphatase (NTPase) activities, that is important for replication (Bazan*et al*., 1989).

NS4

It's coding region codes for two proteins v.i.z, proteins NS4A and NS4B.They are highly hydrophobic transmembrane proteins. NS4a has a major role in replication, assembly, induction of rearrangements. NS4a regulates the ATPase activity of the NS3 helicase activity.

NS5

Flavivirus NS5 non-structural protein consists of two domains, i.e. C-terminal RNA-dependent-RNA polymerase (RdRp) domain and another one is N-terminal MTase where both are acting as enzymes involved in genome replication.

- C-terminal domain is RdRp responsible for the viral genomic RNA replication.
- The N-terminal domain is a methyl transferase belongs to SAM-dependent Mtases family (Zhou *et al*., 2007) which regulates the translation of genome codes.
- Capping of genomes is performed by NS5 in the cytoplasm.

- Methylation of viral RNA cap at guanine N-7 and ribose 2'-O positions by NS5.

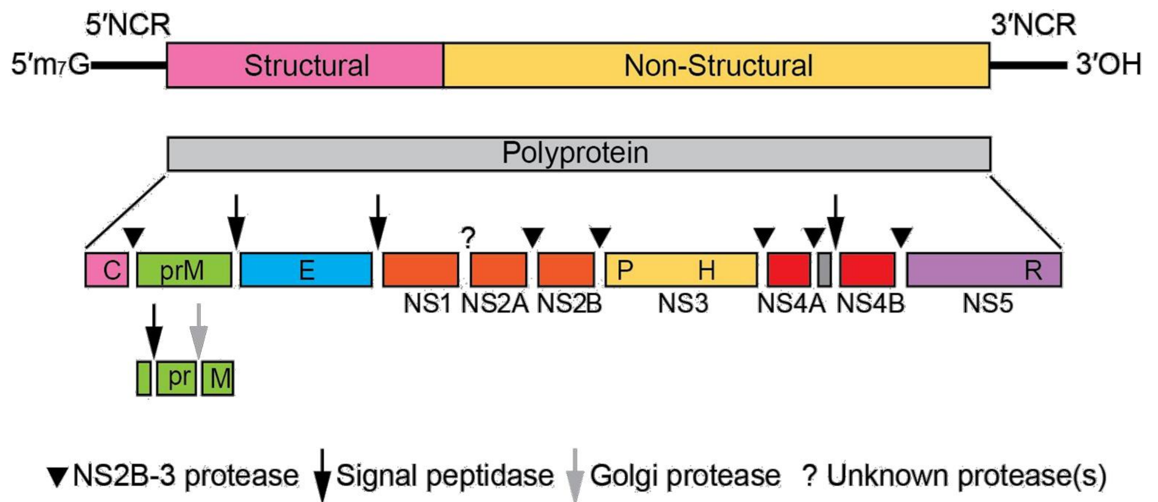


Figure 4: Flavivirus genome organization (talk.ictvonline.org)

2.6 Envelope protein of various flaviviridae members

Blocking cell attachment, endocytosis and membrane fusion shall go a long way in curing diseases caused by flaviviridae members. As E protein leads the viral entry process, it has long been considered a prime target for vaccine and neutralizing antibody development (Pierson *et al.*, 2008; Sanchez *et al.*, 2005 and Stiasny *et al.*, 2007). Hence, E-protein of various flaviviridae members was considered in the present study.

2.6.1 Zika virus envelope protein

The structure of ZIKV comprises an icosahedral shell which consists of 180 copies each of the E protein, atomic weight is 51,000 exists as a homotrimer, glycoprotein of 505 amino acids and structural E protein are present on the outermost shell of the ZV particle. Envelope protein of ZIKV is the main constituent which is involved in membrane fusion, binding of receptor, and host immune recognition .E proteins consist of an E protein complex of three domains that is DI, DII and DIII present on the surface (Shafique S., 2017) and a fourth stem-transmembrane domain acting as an anchor (Figure 5) . Domain I act as a bridge in E protein between two domains, i.e. Domain II and III. The fusion loop (FL), which is present at the tip of DII interacts with the host membrane during membrane fusion. DIII has an important role in fusion as it contains the putative receptor-bindingin most of the flaviviruses (Bressanelli, S .*et al.*, 2004 and Modis Y.*et al*. 2004).

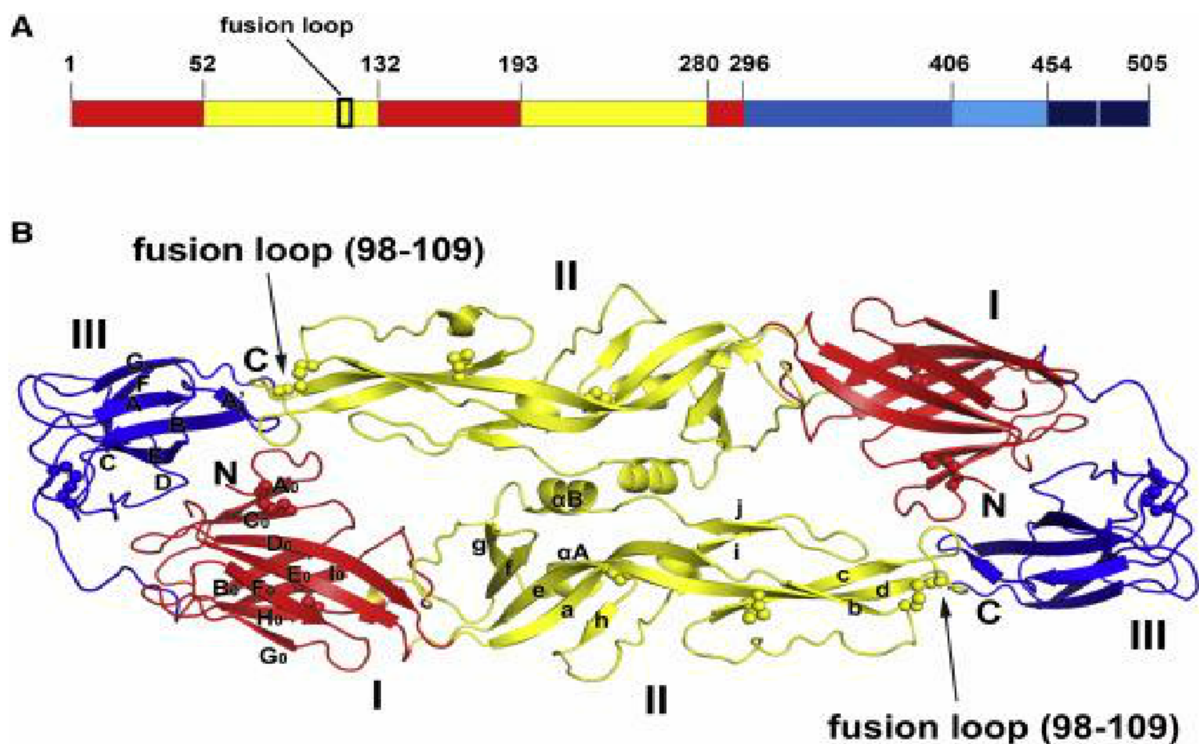


Figure 5: Envelope protein structure of Zika virus (Lindenbech*et al*., 2003)

2.6.2 Dengue virus envelope protein

The DENV E protein is a major part of antigenic structure and consists of 495 amino acids. It is involved in virus cell attachment as well as virus entry into host cells (Rey *et al*., 1995). It exists in dimeric form, a confirmation that changes to trimeric form when exposed to low endosomal pH (Bressanelli *et al*., 2004; Modis *et al*., 2004). The crystal structure of DENV E protein has been resolved during many studies (Modis *et al*., 2003; Zhang *et al*., 2004; Nybakken *et al*., 2006). It appears to be an elongated protein, mainly composed of b-strands andis organized into three domains v.i.z, Domain I (the central domain), Domain II (the dimerization domain) and Domain III (the IgC like domain) (Figure 6).The fusion loop, which is present at the tip of domain II and the fusion loop interacts with the endosomal membrane during the fusion event. Domain III takes part in receptor binding.

The formation of the post-fusion structure of DENV2 and tick-borne encephalitis virus (TBEV) requires the exposure of the proteins to both low pH and liposome (Bressanelli *et al*., 2004; Modis *et al*., 2004). However, DENV1 virus E protein seemed to be able to

13

form post fusion structure in the presence of low pH alone (Nayak *et al*., 2009). The subunits have extensive interactions at both the tip and the base of domain II and the packing interface between domains I and domain III. In the low pH environment of the endosome, the domain II of the E protein flips up, exposing its fusion loop, thus leading to the insertion of the fusion loop into the endosomal membrane. The membrane may then catalyze trimerization, causing the formation of a pre-fusion intermediate. Trimerization, which is then, spreads from the envelope protein fusion tip downwards to envelope domain I at the base of the trimer. Domain III then shifts and rotates, displacing the trimers, which in turn cause the endosomal and viral membrane to fuse. The fusion of membranes may require concerted twisting of a few trimeric E proteins.

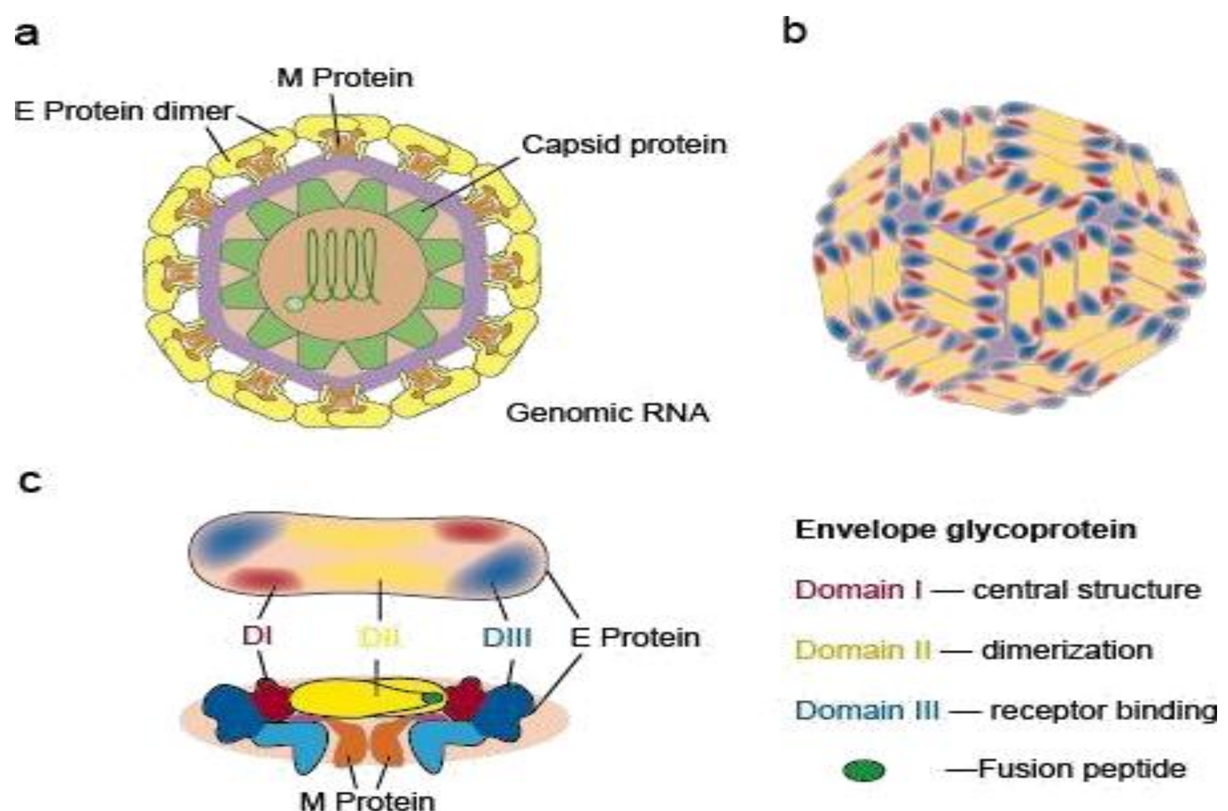


Figure 6: Dengue virus Envelope protein structure (www.creative)

2.6.3 West Nilevirus envelope protein

The E protein in WNV consists of 501 amino acids and exists as a component of the outer shell along with the membrane protein. The E protein can bind to multiple receptor types such as αVβ3 integrin (Chu *et al*., 2004), glycosaminoglycans (Lee *et al*., 2004) or, like E protein of dengue virus, a carbohydrate recognition protein (Tassaneetrithep *et al*., 2003;

Sanchez *et al*., 2003). An example of the latter case was reported in a study by Davis and colleagues where C-type lectin DC-SIGNR acted as a receptor for the glycan on West Nile virus E (Davis *et al*., 2006).

Envelope protein of West nile virus (WNV) has a three-domain structure similar to that of TBEV, DENV-2 and DENV-3. Domain I is located centrally and composed of residues 1-51, 134-195 and 284-297 taking the total amino acid count to 127. Domain II consists of residues 52-133 and 196-283, thus, a total of 170 residues (Figure 7). Domain II which helps in generating the majority of contacts in the DENV and TBEV dimers has been reported to take center stage. The putative fusion loop (residues 98 to 110), hydrophobic sequence, a conserved glycine-rich, that appears necessary for flaviviral fusion (Roehrig *et al*., 1990) is present at the distal end of domain II. Domain III which is an immunoglobulin (Ig)-like domain with seven-strands (Sanchez *et al*., 2003).

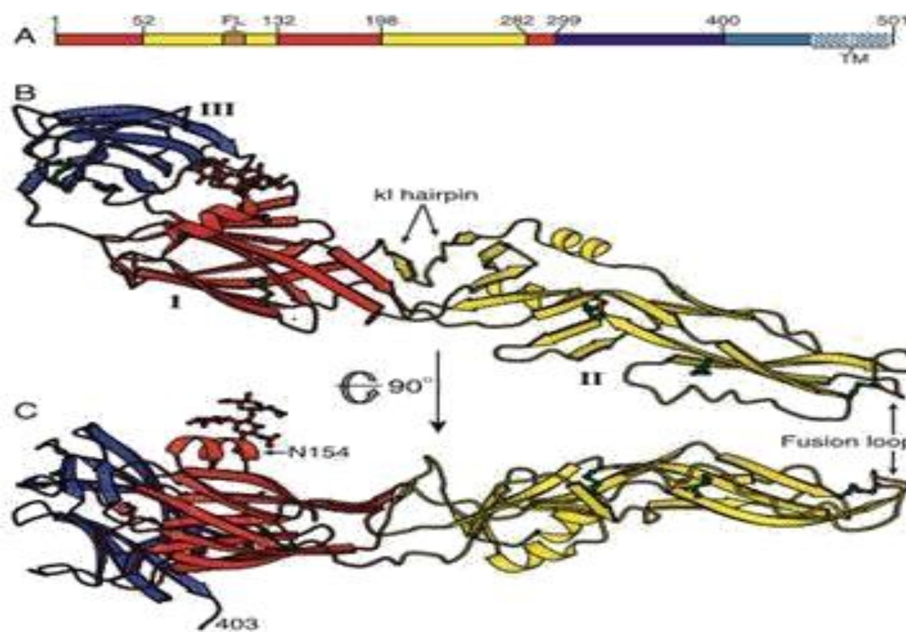


Figure 7: Envelope protein structure of West Nile Virus (jvi.asm.org)

2.6.4 Japanese encephalitis virus envelope protein

The JEV E protein consists of 500 amino acids and as is the case for other family members, is the main structural component of the viral envelope. Japanese Encephalitis virus envelope protein structurally divided into three domains v.i.z., Domain I (with

residues 1-51, 134-195 and 284-296), Domain II(with residues 52-133 and 196-283) and Domain III (with residues 297-395) (Nybakken *et al*., 2006) (Figure 8).

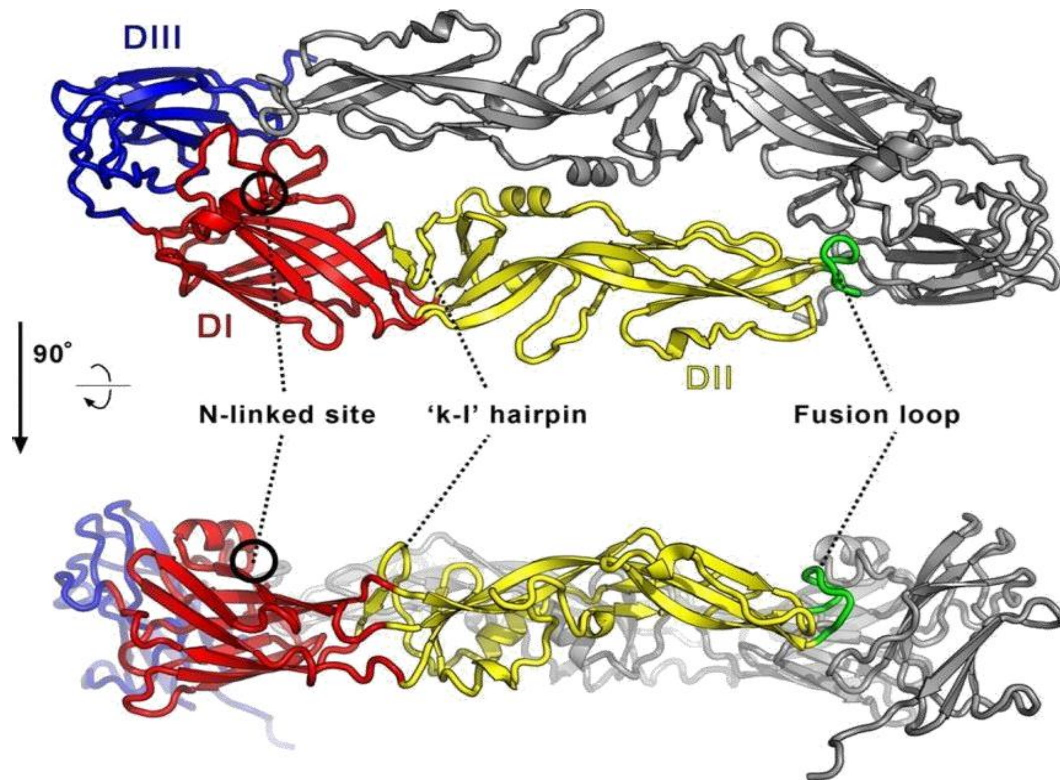


Figure 8: Envelope protein structure of Japanese encephalitis virus (jvi.asm.org)

2.6.5Spondweni virus envelope protein

Spondwenivirus (SPOV) forms one half of the Spondweni serogroup with Zika virus (ZIKV) forming the other half. SPOV E protein consists of 500 amino acids and as for other family members, SPOV E protein is of paramount significance for viral entry and host cell attachment via receptor mediated endocytosis (Dai, Lianpan, 2016).

2.7 Comparison of e-protein of various flaviviridae members

The E protein of different flavivirusesshows approximately 40% identity in the interior region while the outer region differs considerably. This fact is consistent with the absence of cross-protective countermeasures w.r.t distantly related flaviviruses. Furthermore, E-protein can show noticeable differences even within sero-complexes. For example, four serotypes of dengue virus vary by approximately 37% in their E protein amino acid sequence (Lobigs and Diamonds, 2012; Xu *et al*., 2016).

On the other hand, JEV and WNV E proteins exhibit approximately 80% identity thus, forming basis for an idea that sufficiently high antibody titers with the ability to confer immunity is induced by JEV vaccines against diseases caused by both viruses (Lobigs and diamonds, 2012). Though there are many factors to consider the factors of such a cross-protective vaccination approach or development, it still represents an interesting challenge for the scientific community.

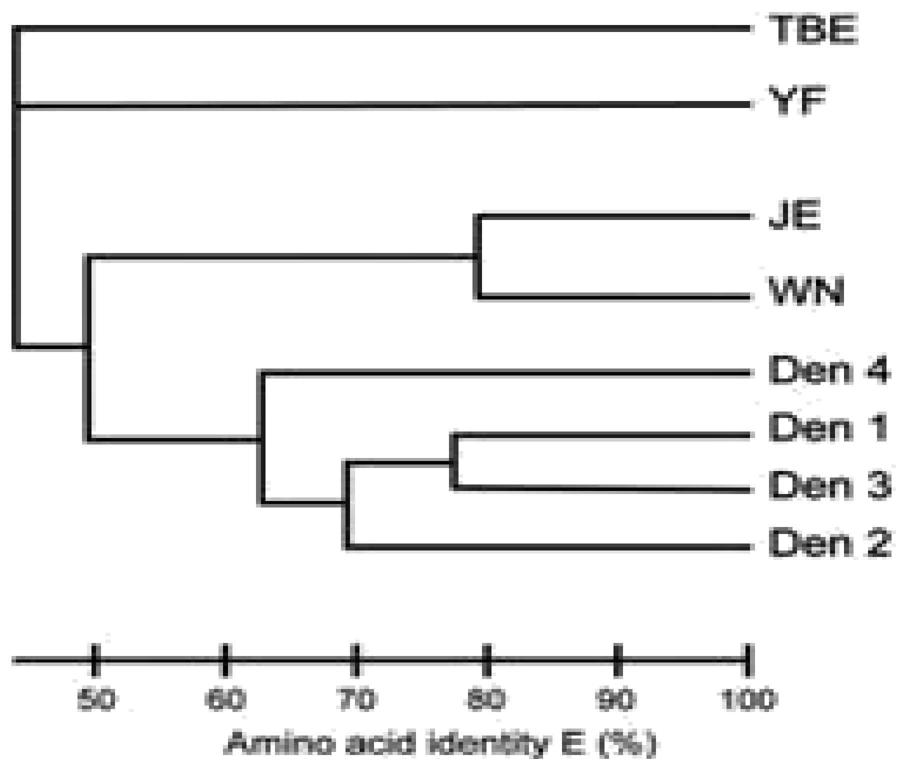


Figure 9: E protein comparison offlaviviruses (Heinz and Stiasny*et al*., 2012)

Table 1: Pairwise sequence comparison of the E proteinsfrom different Flaviviruses (Xiaojun *et al.*, 2016)

E Protein	ZIKA	DENV1	DENV2	DENV3	DENV4	JEV	YFV	WNV	TBEV
ZIKA	100.0	57.8	53.9	57.8	55.6	54.6	42.1	54.0	39.5
DENV1		100.0	68.7	77.6	63.8	50.1	43.0	51.0	39.3
DENV2			100.0	68.3	63.8	47.3	44.8	47.4	38.9
DENV3				100.0	63.2	48.8	42.0	46.7	37.7
DENV4					100.0	47.5	40.5	49.6	40.3
JEV						100.0	43.9	78.4	40.4
YFV							100.0	43.7	41.5
WNV								100.0	40.2
TBEV									100.0

2.8Flaviviridae viruses: Disease and symptoms

General symptoms of flaviviridae infection include fever, body aches, headache, and joint pain. Some of these illness may also cause vomiting and diarrhoea. The diseases caused by Flaviviridae viruses can be fatal. The symptoms and disease of different flaviviruses are mention in table 2. The host, vector and incubation time of flaviviruses are given in table 3.

Table 2: Disease and symptoms of flaviviruses

VIRUS	**DISEASE**	**SYMPTOMS**
ZIKA VIRUS	Microcephaly and Guillian-Barre syndrome	Incomplete brain development and muscle weakness.
DENGUE VIRUS	Dengue hemorrhagic fever (DHF)	Joint and muscle pain ,rash, mild bleeding
WEST NILE VIRUS	An inflammation of brain Encephalitis	Skin rash, swollen lymph nodes, paralysis
JAPANESE ENCEPHALITIS VIRUS	An inflammation of brain called Encephalitis	Fever, headache, seizures
SPONDWENI VIRUS	Spondweni fever	Fever, nausea, myalgia, pruritic rash

Table 3: Host, vector and incubation time of flaviviruses

Disease	Agent[1]	Incubation period	Life cycle	Vector species in Japan[2]	Geographical distribution in Japan	No. reported cases[3]	
						Imported	Domestic
Zika virus infection	Zika virus	3-12 days (mostly 2-7 days)	Human-Mosquito-Human	Aedes albopictus	Areas south of Aomori (as of 2015)	7	–
Chikungunya fever	Chikungunya virus	3-12 days (mostly 3-7 days)	Human-Mosquito-Human			10-20/year	–
Dengue fever	Dengue virus	3-15 days (mostly 3-7 days)	Human-Mosquito-Human			200-250/year in recent years	162 in 2014
Yellow fever	Yellow fever virus	3-6 days	Human-Mosquito-Human			–	–
West Nile fever	West Nile virus	2-14 days	Bird-Mosquito-Human	Culex mosquito species	Nationwide	1 in 2005	–
Japanese encephalitis	Japanese encephalitis virus	1-2 weeks	Pig-Mosquito-Human	Culex tritaeniorhynchus	Nationwide excluding Hokkaido	1 in 2011	1-10/year

2.9 Flaviviridae diseases: Treatment and prevention

No robust preventive and therapeutic measures exist against infections from members of the Flaviviridae as yet. Some targets under consideration for vaccine development against this family include virus attachment to host, uptake of the virus into the cell, viral replication and various proteases involved in the process. (Leyssen *et al*., 2000).

Symptomatic treatment and supportive care and the patients are instructed to take the rest and drink a lot of fluids to avoid dehydration or aggressive fluid management are the best measures available to decrease the mortality rate (Leyssen *et al*., 2000). As mosquitoes are the primary vectors for all these diseases, taking precautions against mosquito bites such as using bug repellents, wearing full sleeved clothes in prone regions goes a long way in evading the unwanted. A study reported that safe sex practices can also decrease the occurrence of disease in humans.

2.10 Vaccines against flaviviridae

Vaccine development has long been a challenge for the scientific community in fight against flaviviridae diseases. As of now, no licensed human vaccines are available in the market against any of the diseases caused by this family, which can confer protection globally against all serotypes of any of the members (Ishikawa *et al*., 2014).

Another factor in vaccine development is the cost and effectiveness. Many attempts have been made at developing vaccines, which have not provided apt coverage of all prone areas. Hence, there is a need to explore paths different from traditional paths of vaccine development (Ishikawa *et al*., 2014).

2.10.1 Vaccines against Zika virus

$CD8^+$ T cells and antibodies are thought to protect against ZIKV infection. Preclinical studies have demonstrated that antibodies suffice for complete protection (Larocca RA *et al*., 2016).

Table 4: Human ZIKV vaccines which are currently in clinical development

Developer	Type of Vaccine	Current Phase
Walter Reed Army Institute of Research(WRAIR)/NIAID	Inactivated	Phase I
National Institute of Allergy and Infectious (NIAID)	DNA	Phase I
InovioGeneONE	DNA	Phase I

2.10.2 Vaccines against dengue virus

The biggest challenge in developing a vaccine against DENV is to design a globally protective vaccine which will be protective against serotype (I-IV) of dengue virus to decrease the risk of immune-mediated enhancement. The first licensed vaccine (named Dengvaxia; CYD-TDV, a tetravalent live attenuated vaccine) was developed in 2015by Sanofi Pasteur. Itwas authorized for use currently and 11 countries have given it a go ahead (Guy B *et al*., 2011). In two large phase III clinical trials, an efficacy of 66% was observed even though the serotype-specific efficacy for DENV2 was pathetic (~43%) (Villar L *et al*., 2015).

Table 5: Vaccines against dengue virus that are licensed or in different clinical stage

Developer	Type of Vaccine	Current Phase
Sanofi Pasteur	Live attenuated	Licensed (Guy B *et al*., 2011)
National Institute of Allergy and Infectious (NIAID)	Live attenuated	Phase III
United states(U.S.) Naval Medical Research Center	Recombinant subunit Deoxyribonucleic acid(DNA)	Phase I

2.10.3 Vaccines against west nile virus (WNV)

2003 and 2012 witnessed the two largest outbreaks of WNV in recent times, pointing towards a need to develop vaccines against it (Diamond MS *et al.*, 2003). T cells were shown to take centre stage in fighting and limiting the spread of WNV (Shrestha, 2004). On the other hand, the major role of T cells is protection during infection has been questioned in many studies (Szretter KJ *et al.*, 2012).

Table 6: Human West Nile Virus vaccines which are currently in clinical development

Developer	Type of Vaccine	Current Phase
National Institute of Allergy and Infectious (NIAID)	Live attenuated	Phase I
Sanofi Pasteur	Live attenuated	Phase II
Vical/NIAID	Recombinant subunit DNA	Phase I

2.10.4 Vaccines against Japanese encephalitis virus (JEV)

The first JEV vaccine is an inactivated vaccine which was prepared in mouse brain. The strain for this inactivated vaccine was Nakayama was developed in Japan in 1954(Ishikawa *et al.*, 2010). Since 1989, in place of Nakayama strain, and the Beijing-1 strain exhibiting immune responses against broader strains has been used.

2.11 Peptide vaccines

Peptide as vaccines candidates has shown recent interest where the peptides are mapped to contain the T and B cell epitopes with potential to induce specific immune responses. One of the approaches used to design peptide vaccines that combines the immunoinformatic prediction with rigorous experimental validation and hence, is considered as an interdisciplinary approach (Flower *et al.*, 2013). Peptides that are used as vaccine candidates are usually 20-30 amino acids long. These peptide lengths are considered for the activation of cellular and humoral responses. B cell antigenic determinant of a target molecule can also be combined with a T-cell epitope to obtain a vaccine candidate with higher immunogenicity. In epitope based vaccine, immune response induced by an

immunogen is not directed against the whole molecule, but it is against a specific region (sequence) of protein which is an epitope. The primary epitope-based immunization was made in 1985. They presented recombinant DNA and expressed epitopes in *Escherichia*

Table 7: Human JEV vaccines which are currently in clinical development

Developer	Type of Vaccine	Current Phase
Central Research Institute, South Korea Adimmune corporation (Taiwan),India Government Pharmaceutical Organization (Thailand)	Inactivated	In many countries cell culture-derived vaccines is shifting towards
Chengdu Institute of Biological Products,China	Live-attenuated	approved in many countries like china, Cambodia, north Korea, India
Sanofi Pasteur (France)	Live-attenuated chimeric	prequalified by the WHO

coli against cholera (Dermime*et al*., 2004) (Meloen *et al*., 2001).A recent study has identified conserved peptides containing multiple T-cell epitopes of matrix 1 protein in H1N1 flu using immunoinformatics and then validated immunogenic response in PBMC (Lohia*et al*., 2015, Lohia *et al*., 2017).Thus, development of epitope based vaccines for T and B lymphocytes is feasible. Moreover, peptide-based epitope ensemble vaccines offer various advantages over traditional whole-organism vaccines. They solely focus on the immune response of relevant epitopes and hence, help in avoiding non desirable effects like autoimmunity. Another advantage of such vaccines is that they induce allergen specific tolerance (Hailemichael *et al*., 2013). These vaccines represent no danger of change or inversion and almost no risk of contamination by harmful or pathogenic substances. They are comparatively easy to deliver than a regular vaccine and are also chemically stable.

Peptide-based vaccines have proved to be a boon for the treatment of life threatening diseases like hepatitis C and cancer (Hailemichael *et al*., 2013). In a study, metadherin based peptide vaccine against cancer has been carried out based on immunoinformatics approach (Dhiman *et al*., 2016). Even though epitope-based vaccines can be long-range and broad spectrum immunizations, yet with the utilization of small molecules as peptides, an issue of low immunogenicity as compared to traditional vaccines is there.

2.12 Immunoinformatics

Immunoinformatics (computational immunology) is a sub-branch of bioinformatics that makes use of data management and computational tools to solve immunological problems (Brusic *et al*., 2004). Conventional methods involve pathogen cultivation and subsequent protein extraction which is very tedious, cost ineffective and needs lots of time. Immunoinformatics investigation stresses on finding the potential B-and T-cell epitopes. This approach accelerates the time and brings down the cost required for research centre examination of pathogen related gene products (Patronov *et al*., 2013). By utilizing these computational tools and data, an immunologist can analyze the sequence areas with potential sites for binding, which thus prompts the advancement of new vaccines. The approach of reverse vaccinology is used to identify the potential immunogenic peptide regions in a pathogen.

3. MATERIAL AND METHODS

3.1 Sequence retrieval

Unique envelope protein sequences belonging to pathogenic strains of four flaviviridae members Dengue, West Nile Virus, Japanese Encephalitis virus, Spondweni virus were downloaded from The Virus Pathogen Database and Analysis Resource. This database is a hub of virus-centred data and contains an integrated information and analysis tools for many virus families that cause infection in humans. It is a great measure taken by the NIAID within the US National Institutes of health (NIH) which provides integrated online resources for data on different pathogens. ViPR is a freely available online database that provides access to various type of information including annotation, sequence entries, immunogenic epitopes, and 3D structures.

Complete E protein sequences and genome with human as a host were downloaded in FASTA format and saved in Microsoft office word. Duplicate genome sequences or incomplete protein sequences were avoided.

3.2 Multiple sequence alignment

Multiple sequence alignment is done utilizing MUSCLE tool (http://www.ebi.ac.uk/Tools/msa/muscle). MUSCLE acronym stands for Multiple Sequence Comparison by Log-Expectation. MUSCLE is a computer based program for making numerous arrangements of protein sequences. Components of the algorithm incorporate quick separation estimation utilizing kmer tallying, progressive alignment utilizing the score of log-desire, and refinement utilizing restricted partitioning which is tree dependent. MUSCLE is asserted to accomplish both better accuracy and good speed over ClustalW2 or T-Coffee (Edgar RC., 2004). It is licensed as a public domain.

The constraint of this tool is that it can only align 500 sequences of more than 350 amino acid length at a time. The results of MUSCLE were saved in FASTA format. The number of sequence retrieved was more than 500 and hence, another tool (CLUSTAL W) has been used.

3.3 Conservancy analysis

Consensus sequences obtained after multiple sequence alignment were used to locate the position of differentpeptide sequences. Percentage identity was calculated manually based

on the presence of identical/different amino acid at the same position between two peptides. Threshold for identity was taken as >=70%.

3.4 Peptide screening

3.4.1 Peptide Match

Protein sequence databases play a vital role in the different area of proteomics. The Protein Information Resource (PIR) (Wu *et al.*, 2003) has provided a much used and efficient peptide match service freely to online users since a decade. This service helps in detecting the presence of peptides similar to the query sequence.

More than one function for both, input query and output navigation is provided by Peptide Match web interface. A query sequence is provided as an input by user and then, a particular organism/s or the complete database is selected as per requirement for finding matches similar to the query. To perform the proper function, a user can provide the input sequence as by the name of an organism of complete proteome of UniProt, set of organisms from the reference proteomes ofUniProtor the taxonomic ID of the organism.

3.4.2 Algpred

With the help of AlgPred,allergenicity of the predicted peptides was determined. It finds allergic peptides based onscreening of IgE epitopes in sequence of query protein. It isbased on specific criteria's that includes epitope profiles, mapping of IgE epitopes and search of similarity against IgE epitopes, structure profiles and if protein have any motif that is assign a allergen protein using MAST (Ivanciuc *et al*., 2003).These IgE epitopes were scanned at a fixed percent of identity (PID) and also scanned against sequence dataset of allergic and non-allergic proteins. It is also scanned on different percent of identity depend upon IgE epitopes length.

With the help of a window of amino acids, if a protein/peptide possesses 35% identity or six consecutive identical amino acids are found when compared to an established allergen (www.fao.org.), that expressed peptide or protein will be a selected as a potential allergen. In the current study, when a protein or peptide is predicted as an allergen if it has one or more IgE epitopes.

3.4.3 ToxinPred

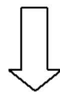
For identification and screening of toxic peptides in a dataset of random peptides or non-toxic which is obtained from SwissProt and the translation of European Molecular Biology Laboratory (EMBL) nucleotide sequences databases (Gupta *et al.*, 2013), an *in silico* approached tool is used that is "ToxinPred". The main dataset used in this method consists of 1805 toxic peptides (<=35 residues).This tool helpsin identifying and analyzingtoxicity of peptides/proteins and designing peptides with least toxicity. In proteins, it is useful to discover toxic regions and also helpful in peptide/protein-based drug discovery (http://crdd.osdd.net/raghava/toxinpred/) by providing momentum. All predications were that high specificity would be maintained, which we can define by a stringent threshold value.

3.5 Mapping of peptide fragments

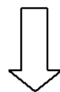
The 3D envelope protein structures of all viruses under study were obtained from PDB. The multimeric structures were edited to monomeric structures with the help of Discovery Studio v4.1 visualizer. E protein structure for SPOV was not available in the database.

Procedure followed for labelling the peptides (in brief):

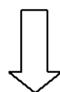
Download the E protein structure with a good resolution from PDB



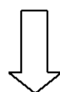
Open the multimeric structure of E-protein in discovery studio visualization tool 4.1



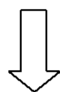
Go to the hierarchy option and remove the extra chains



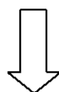
Go to sequence option and then show sequence



Select the whole sequence and change the colour to white



Select the peptides to be located and change their colour



Save the image

Table 8: PDB id's used to locate identified peptides

VIRUS	PDBid	Resolution (Å)
ZIKA VIRUS	5JHM	2 Å
DENV I	3G7T	3.5 Å
DENV II	1OAN	2.75 Å
DENV III	1UZG	3.5 Å
DENV IV	3UAJ	3.23 Å
WEST NILE VIRUS(WNV)	2HG0	3 Å
JAPANESE ENCEPHLAITIS VIRUS(JEV)	5MV1	2.25 Å
SPONDWENI VIRUS	NA	

3.6 CABSdock analysis

Docking of Class I epitopes was carried out with six HLA molecules with the help of CABSdock (Kurcinski et al., 2015) Unlike AutodockVina, there is no need to define the binding groove with the help of a grid to perform docking with the help of CABSdock. Also, CABSdock allows full flexibility of the peptide.

The PDBid for the sixHLA molecules and the epitope sequences were provided as input in the online server.

Table 9: PDB ids with corresponding HLA allele and resolution

PDBid	Corresponding HLA allele	Resolution (Å)
3C9N	B*1501	1.87 Å
3MRE	A2	1.1 Å
2BVP	HLA-B5703	1.35 Å
3B08	A1	1.7 Å
3SKO	B8	1.6 Å
3RL1	A*0301	2 Å

4. RESULTS AND DISCUSSION

4.1 Peptides selected for this study

Envelope protein sequence of differentflaviviruses [ZIKA, Dengue (DENV), West nile virus (WNV), Spondweni virus (SPOV) and Japanese encephalitis virus (JEV)] were reported to have partial identity .In previous study, seven peptides containing multiple epitopes of Zika Virus Envelope protein were identified with the help of various epitope prediction and docking tools (Table 10).In a bid to develop a cross protective vaccine effective against various flaviviridae members, these identified Zika glycoprotein peptides were taken as a reference to identify their presence in four other members (DENV, WNV, SPOV and JEV).

Table10: Selected peptides containing multiple T cell epitopes of Zikavirus envelope protein

Peptides	Peptides containing multiple $CD8^{+}$& $CD^{+}4$ T cell epitopes
P1	QSDTQYVCKRTLVD
P2	GLFGKGSLVT
P3	SIQPENLEYRIMLSVHGSQHS
P4	VEITPNSPRAEA
P5	LKCRLKMDKLRLKGVSYSL
P6	VLGDTAWDFGSV
P7	FKSLFGGMSWFSQ

4.2 Sequence retrieval

The envelope glycoprotein sequences of four flaviviruses (DENV, WNV, SPOV and JEV) were obtained from Virus Pathogen Database and Analysis Resource (ViPR). Duplicate and/or incomplete protein sequences were removed and only unique, complete envelope protein sequences with humans as hostwere downloaded in FASTA format (Table 11) and saved in Microsoft office word.

4.3 Sequence comparison

Multiple sequence alignment (MSA) was carried out of the obtained sequences with the help of MUSCLE tool to obtain consensus envelope protein sequence of all flaviviruses under consideration. The consensus sequence of all viruses was compared one by one with the consensus sequence of Zika virus to locate the presence of previously identified Zika envelope protein peptides containing multiple epitopes.

The color codes for envelope protein peptides v.i.z., P1, P2, P3, P4, P5, P6 and P7 in consensus sequences of flaviviruses are red, green, brown, orange, magenta, blue and sky blue respectivelyas shown below.

Table 11: Total number of sequences available in the database and the number of unique sequences downloaded

Virus	**Total number of sequences**	**Number of unique sequences**	**Time frame**
Zika(Reference)	462	105	1944 to 28/05/18
Dengue			
Serotype I	1656	437	1944 to 28/05/18
Serotype II	1237	342	1944 to 28/05/18
Serotype III	811	228	1956 to 28/05/18
Serotype IV	191	82	1956 to 28/05/18
West Nile Virus	140	57	1953 to 28/05/18
Spondweni Virus	1	1	
JapaneseEncephalitisVirus	16	13	1990 to 28/05/18

Zika virus

IRCIGISNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMA
EVRSYCYEASISDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLF
GKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDE
NRAKVEITPNSPRAEATLGGFGILGLDCEPRTGLDFSDLYYLTMNNKHWLVHKE
WFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTAL
AGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLH
GTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLE
LDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDF
GSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISL
MCLALGGVLIFLSTAVSA

Dengue virus

Serotype I

MRCVGIGNRDFVEGLSGAPWVDVVLEHGSCLSNMAKNKPTLDIDFFKTEVTNPA
VLRKLCIEAKISNTTTDSRCPTQGEATLVEEQDANFVCRRTFVDRGWGNGCGLFG
KGSLLTCAKFKCVTKLEGKIVQYENLKYSVIVTVHTGDQHQVGNETTEHGTIATI
TPQAPTSEIQLTDYGALTLDCSPRTGLDFNEMVLLTMKEKSWLVHKQWFLDLPLP
WTSGASTSQETWNRQDLLVTFKTAHAKKQEVVVLGSQEGAMHTALTGATEIQM
SGTTTIFAGHLKCRLKMDKLTLKGVSYVMCTGSFKLEKEVAETQHGTVLVQVKY
EGTDAPCKIPFSTQDEKGVTQNGRLITANPIVTDKEKPVNIETEPPFGESYIVIGAGE
KALKLSWFKKGSSIGKMFEATARGARRMAILGDTAWDFGSIGGVFTSVGKLVHQ
VFGTAYGVLFSGVSWTMKIGIGILLTWLGLNSRSTSLSMTCIAVGMVTLYLGVMV
QA

Serotype II

IRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATL
RKYCIEAKLTNTTTESRCPTQGEPSLKEEQDKRFVCKHSMVDRGWGNGCGLFGK
GGIVTCAMFTCKKNMEGKIVQPENLEYTIVVTPHSGEEHAVGNDTGKHGMEIKV
TPQSSITEAELTGYGTVTMECSPRTGLDFNEMVLLQMENKAWLVHRQWFLDLPL
PWLPGADKQESNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQ
MSSGNLLFTGHLKCRLRMDKLQLKGMSYSMCTGKFKVVKEIAETQHGTIVIRVQ
YEGDGSPCKIPFEIMDLEKRYVLGRLITVNPIVTEKDSPVNIEAEPPFGDSYIIIGVEP

GQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQ
VFGAIYGVAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLVLVGIVTLYLGVMVQ
A

Serotype III

MRCVGIGNRDFVEGLSGATWVDVVLEHGGCVTTMAKNKPTLDIELQKTEATQL
ATLRKLCIEGKITNITTDSRCPTQGEAALPEEQDQNYVCKHTYVDRGWGNGCGLF
GKGSLVTCAKFQCLEPIEGKVVQHENLKYTVIITVHTGDQHQVGNETQGVTAEIT
PQASTTEAILPEYGTLGLECSPRTGLDFNEMILLTMKNKAWMVHRQWFFDLPLP
WTSGATTETPTWNRKELLVTFKNAHAKKQEVVVLGSQEGAMHTALTGATEIQN
SGGTSIFAGHLKCRLKMDKLELKGMSYAMCTNTFVLKKEVSETQHGTILIKVEYK
GEDAPCKIPFSTEDGQGKAHNGRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGD
NALKINWYKKGSSIGKMFEATARGARRMAILGDTAWDFGSVGGVLNSLGKMVH
QIFGSAYTALFSGVSWVMKIGIGVLLTWIGLNSKNTSMSFSCIAIGIITLYLGAVVQ
A

Serotype IV

MRCIGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTAKEV
ALLRTYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRRDVVDRGWGNGCGLF
GKGGVVTCAKFLCSGKITGNLVQIENLEYTVVVTVHNGDTHAVGNDTSNHGVTA
TITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMKMKKKTWLVHKQWFLDLP
LPWTAGADTSEVHWNNKERMVTFKVPHAKRQDVTVLGSQEGAMHSALAGATE
VDSGDGNHMFAGHLKCKVRMEKLRIKGMSYTMCSGKFSIDKEMAETQHGTTVV
KVKYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPFAENTNSVTNIELEPPFGDSYIVI
GVGDSALTLHWFRKGSSIGKMFESTYRGARRMAILGETAWDFGSVGGLFTSLGK
AVHQVFGSVYTTMFGGVSWMIRILIGFLVLWIGTNSRNTSMAMTCIAVGGITLFL
GFTVQA

West nile virus (WNV)

FNCLGMSNRDFLEGVSGATWVDLVLEGDSCVTIMSKDKPTIDVKMMNMEAANL
ADVRSYCYLASVSDLSTKAACPTMGEAHNEKRADPAFVCKQGVVDRGWGNGC
GLFGKGSIDTCAKFACTTKATGWIIQKENIKYEVAIFVHGPTTVESHGNYSTQMG

ATQAGRFSITPSAPSYTLKLGEYGEVTVDCEPRSGIDTSAYYVMSVGAKSFLVHRE
WFMDLNLPWSSAGSTTWRNRETLMEFEEPHATKQSVVALGSQEGALHQALAGA
IPVEFSSNTVKLTSGHLKCRVKMEKLQLKGTTYGVCSKAFKFAGTPADTGHGTV
VLELQYTGTDGPCKVPISSVASLNDLTPVGRLVTVNPFVSVATANSKVLIELEPPF
GDSYIVVGRGEQQINHHWHKSGSSIGKAFTTTLRGAQRLAALGDTAWDFGSVGG
VFTSVGKAIHQVFGGAFRSLFGGMSWITQGLLGALLLWMGINARDRSIAMTFLA
VGGVLLFLSVNVHA

Japanese encephalitis virus (JEV)

FNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPTLDVRMINIEASQLAE
VRSYCYHASVTDISTVARCPTTGEAHNEKRADSNYVCKQGFTDRGWGNGCGLF
GKGSIDTCAKFSCTSKAIGRTIQPENIKYEVGVFVHGTTTSENHGNYSAQVGASQA
AKFTVTPNAPSITLKLGDYGEVTLDCEPRSGLNTEAFYVMTVGSKSFLVHREWFH
DLSLPWTSPSSTAWRNRELLMEFEEAHATKQSVVALGSQEGGLHQALAGAIVVE
YSSSVKLTSGHLKCRLKMDKLALKGTTYGMCTEKFSFAKNPADTGHGTVVIELT
YSGSDGPCKIPIVSVASLNDMTPVGRLVTVNPFVATSSSNSKVLVEMEPPFGDSYI
VVGRGDKQINHHWHKAGSTLGKAFSTTLKGAQRLAALGDTAWDFGSIGGVFNSI
GKAVHQVFGGAFRTLFGGMSWITQGLMGALLLWMGVNARDRSIALAFLATGGV
LVFLATNVHA

Spondweni virus

IRCIGIGNRDFIEGMSGGTWVDIVLEHGGCVTVMSNDKPTLDFELVTTTASNMAE
VRSYCYEANISEMASDSRCPTQGEAYLDKMADSQFVCKRGYVDRGWGNGCGLF
GKGSIVTCAKFTCVKKLTGKSIQPENLEYRVLVSVHASQHGGMINNDTNHQHDK
ENRARIDITASAPRVEVELGSFGSFSMECEPRSGLNFGDLYYLTMNNKHWLVNRD
WFHDLSLPWHTGATSNNHHWNNKEALVEFREAHAKKQTAVVLGSQEGAVHAA
LAGALEAESDGHKATIYSGHLKCRLKLDKLRLKGMSYALCTGAFTFARTPSETIH
GTATVELQYAGEDGPCKVPIVITSDTNSMASTGRLITANPVVTESGANSKMMVEI
DPPFGDSYIIVGTGTTKITHHWHRAGSSIGRAFEATMRGAKRMAVLGDTAWDFG
SVGGMFNSVGKFVHQVFGSAFKALFGGMSWFTQLLIGFLLIWMGLNARGGTVA
MSFMGIGAMLIFLATSVSG

4.4 Peptide identity analysis

4.4.1 Percentage identity to previously identified immunogenic Zika peptides

Percentage identity of the four flavivirus peptides to previously identified Zika virus peptides was analyzed manually. The threshold identity for selecting peptides was kept as ≥70%. Interestingly, four out of seven Zika virus multi-epitopic peptides have remarkableidentity in four viruses (Table 12).Twenty fourdifferentpeptides fragments belonging to various flaviviridae members were obtained based on the set threshold (Table 13). Only two peptides (DENV SIV P5 and JEV P7) showing less than 70% identity to Zika peptide were selected. DENV SIV P5 was selected as in all other serotypes of the Dengue virus showed greater than 70% identity for this peptide. JEV P7 was found to close to 70 %.Four peptides (P2, P5, P6 and P7) were observed to present a stronger case based on their percentage identity in all the five flaviviruses. Moreover, as seen from Table, P2 and P6 have shown most conservancy amongst the flaviviruses and hence, can be considered as strong vaccine candidates.

Table 12: Comparison of peptides showing the best percentage identity

Virus	Percentage Identity			
	P2	**P5**	**P6**	**P7**
Dengue Serotype I	90	84.2	83.3	-
Dengue Serotype II	80	78.9	83.3	-
Dengue Serotype III	100	78.9	91.7	-
Dengue Serotype IV	80	57.9	83.3	-
West Nile Virus	80	-	91.7	76.9
Spondweni Virus	80	84.2	100	84.6
Japanese Encephalitis Virus	80	73.7	83.3	69.2

Table 13: Peptides in different flaviviruses

Virus	P2	P5	P6	P7
Zika (Reference)	GLFGKGSLVT	LKCRLKMDKLRLKGVSYSL	VLGDTAWDFGSV	FKSLFGGMSWFSQ
Dengue Serotype I	GLFGKGSLLT	LKCRLKMDKLTLKGVSYVM	ILGDTAWDFGSI	YGVLFSGVSWTMK
Dengue Serotype II	GLFGKGGIVT	LKCRLRMDKLQLKGMSYSM	ILGDTAWDFGSL	YGVAFSGVSWTMK
Dengue Serotype III	GLFGKGSLVT	LKCRLKMDKLELKGMSYAM	ILGDTAWDFGSV	YTALFSGVSWVMK
Dengue Serotype IV	GLFGKGGVVT	LKCKVRMEKLRIKGMSYTM	ILGETAWDFGSV	YTTMFGGVSWMIR
West Nile Virus	GLFGKGSIDT	LKCRVKMEKLQLKGTTYGV	ALGDTAWDFGSV	FRSLFGGMSWITQ
Japanese Encephalitis Virus	GLFGKGSIDT	LKCRLKMDKLALKGTTYGM	ALGDTAWDFGSI	FRTLFGGMSWITQ
Spondweni Virus	GLFGKGSIVT	LKCRLKLDKLRLKGMSYAL	VLGDTAWDFGSV	FKALFGGMSWFTQ

4.5 Peptide screening

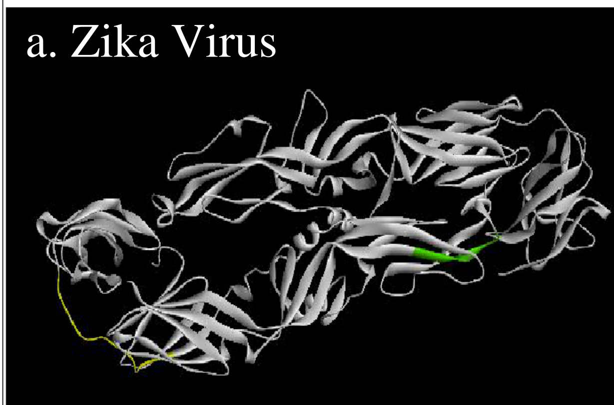
In order to avoid any similarity of the peptides with functional human protein, Peptide match analysis was performed. Further, allergenicity of the identified peptides was predicted sing an online server AlgPred.ToxinPredwas used to identify toxic peptides.

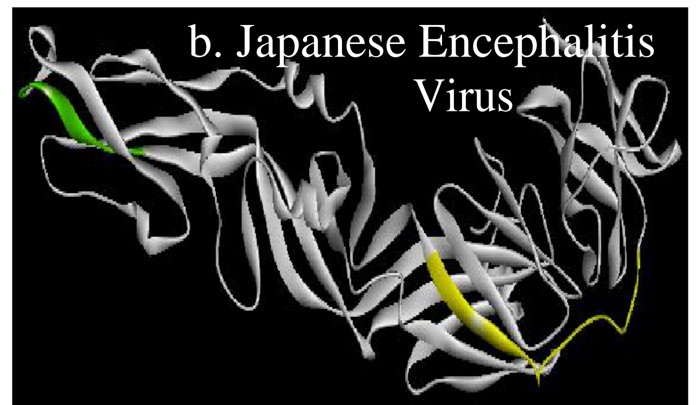
It was interesting to find that all peptides were non-allergen, non-toxic and there were no peptides match of flaviviruses that cause autoimmunity in host.

4.6 Mapping of peptide fragments

The envelope protein structure of all flaviviruses (except SPOV) was obtained from Protein Data Bank (PDB). Some of the proteins were found to have a multimeric structure. Therefore, the PDB structure of such proteins was edited to remove the multimeric structures leaving only monomers using Discovery Studio v4.1 visualizer.

Peptides except P2 and P5 could not be mapped in structuresas the corresponding sequences were missing in the structures.





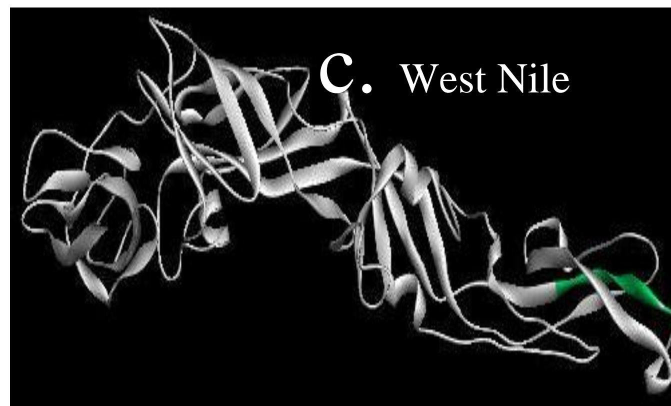
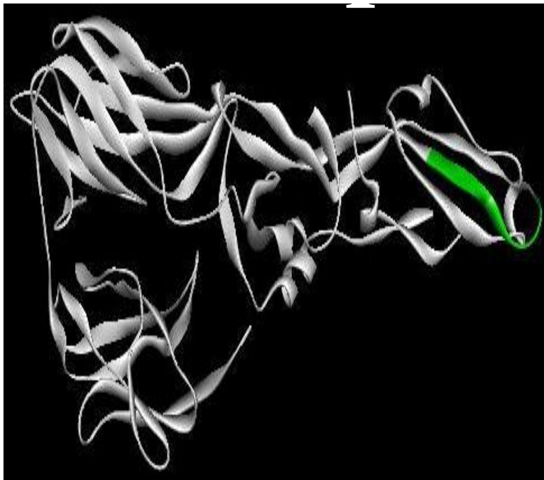


Figure 10: Mapping of peptides on 3 D structure of Envelope protein**.** Peptide fragments P2 and P5 are color coded as green and yellow respectively.a) Mapping of P2 and P5 on Zika virus envelope protein b) P2 and P5 on JEV envelope protein c) P2 on WNV envelope protein.

38

Dengue Serotypes (I-IV)



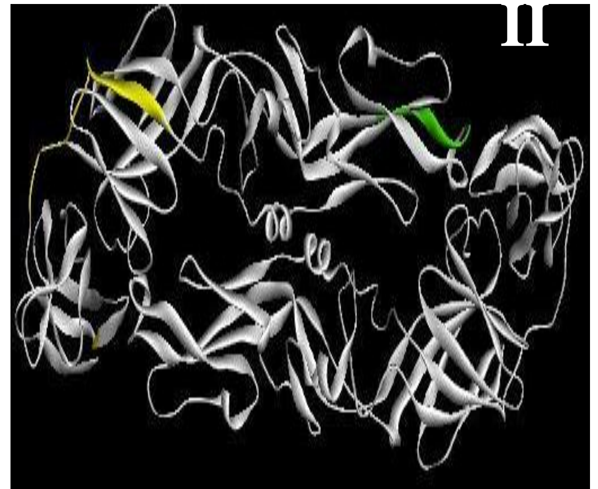


Figure 11: Mapping of peptides on 3D structure of Envelope protein of Dengue virus serotypes. Peptide fragments P2 and P5 are colour coded as green and yellow respectively. P2 on Serotype I, P2 and P5 on Serotype II, P2 on Serotype IIIand P2 and P5 on Serotype IV

4.7 Zika HLA-peptide interaction analysis: CABSdock

CABS dock analysis present favourable results and support the HLA-interaction findings via AutodockVina carried in the previous study. Due to time constraints, only Class I epitopes present in the final peptides and 6 HLA molecules could be considered. Average RMSD values for the epitopes belonging to same peptide were considered for final analysis. Only values ≤5 have been considered.RMSD values < 3 are considered high quality predictionswhile 3 ≤ RMSD ≥ 5.5 moderate quality predictions (Blaszczyk et al., 2016). The average of all four peptides were found to be close to RMSD value of native peptides (Figure 12). In most of the cases, the average RMSD value was found to be less than 3 representing the strong binding potential of these peptides with HLA molecules (Figure 12). The best docking pose of peptide-HLA is shown in figure 13.

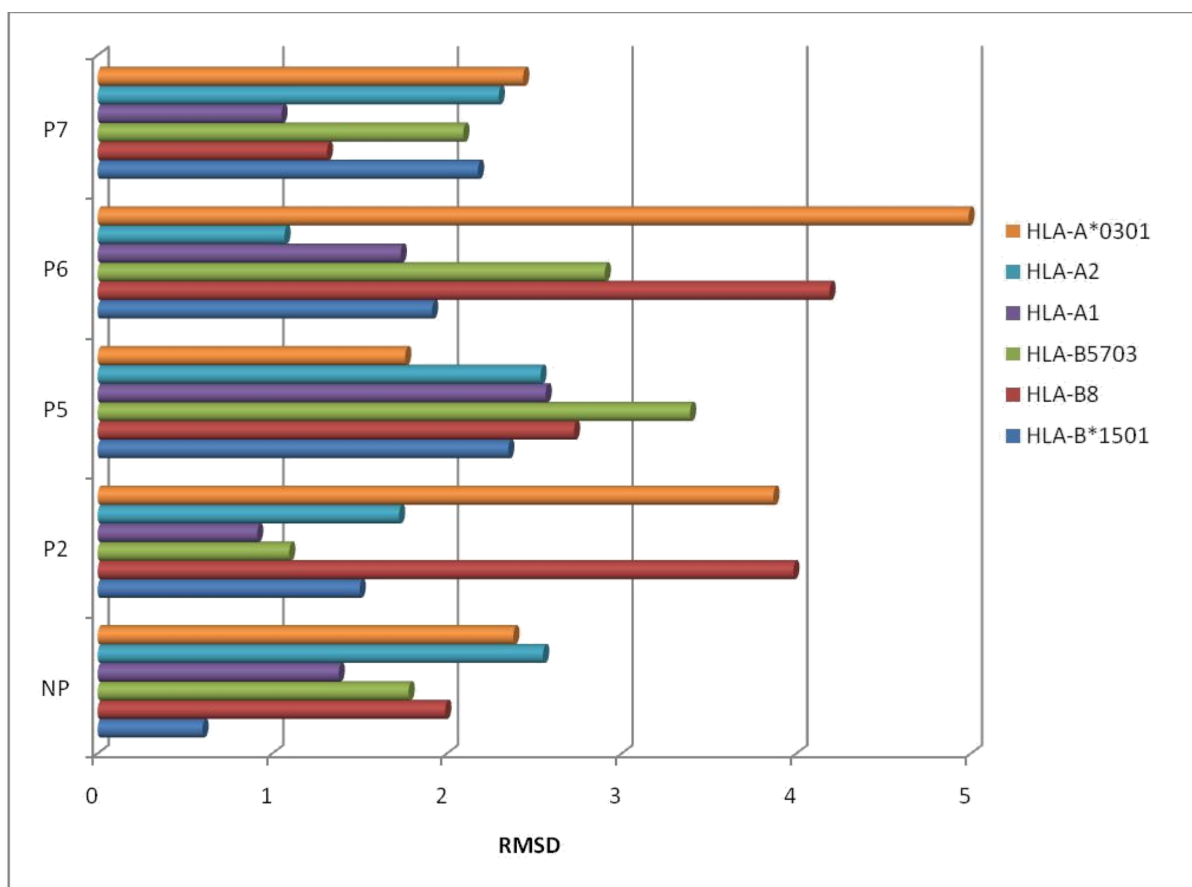


Figure 12:RMSD values for native and peptides (P2, P5, P6 and P7) of Zika Envelop protein.

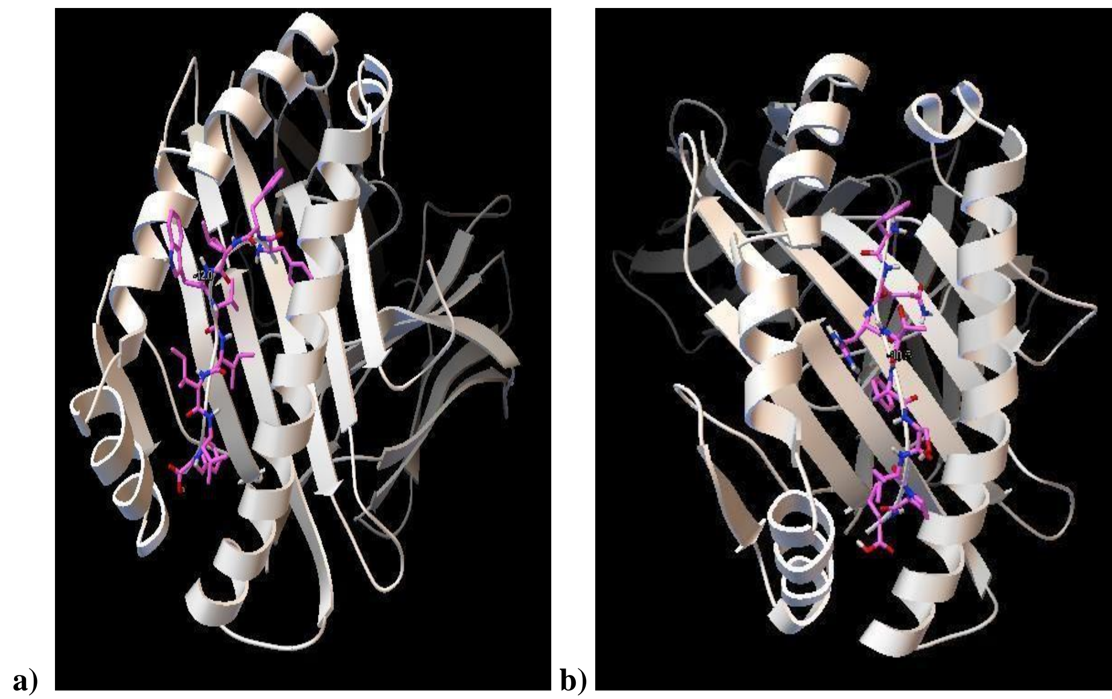


Figure 13: Best docking poses obtained via CABSdock. a) DTAWDFGSV epitope with HLA-A2 b) LEYRIMLSV epitope with HLA-B5703

5. CONCLUSION

Computational approach in immunology has shown success in mapping the epitopes and analyzing the molecular interaction of peptides with HLA molecules to finda vaccine candidate against different viruses. The protein sequence of different flaviviruses reported to share homology among themselves that give a direction to develop cross-protective vaccine. Four peptides containing multiple epitopes of Zika virus Envelop protein were identified which has shown their identity (≥ 70%) in other four flavivirus (Dengue, West Nile Virus, Japanese encephalitis virus, andSpondweni virus). These peptide have also shown strong binding affinity with different HLA molecules.

REFERENCES

1. Anfasa, F., Siegers, J. Y., van der Kroeg, M., Mumtaz, N., Raj, V. S., de Vrij, F. M., & Reusken, C. (2017). Phenotypic differences between Asian and African lineage Zika viruses in human neural progenitor cells. mSphere, 2(4), e00292-17.
2. Bazan, J. F., & Fletterick, R. J. (1989). Detection of a trypsin-like serine protease domain in flaviviruses and pestviruses. Virology, 171(2), 637-639.
3. Bray, M., & Lai, C. J. (1991). Dengue virus premembrane and membrane proteins elicit a protective immune response. Virology, 185(1), 505-508.
4. Bressanelli, S., Stiasny, K., Allison, S. L., Stura, E. A., Duquerroy, S., Lescar, J., & Rey, F. A. (2004). Structure of a flavivirus envelope glycoprotein in its low‐pH‐induced membrane fusion conformation. The EMBO journal, 23(4), 728-738.
5. Brusic, V., Bajic, V. B., & Petrovsky, N. (2004). Computational methods for prediction of T-cell epitopes—a framework for modelling, testing, and applications. Methods, 34(4), 436-443.
6. Chu, J. J. H., & Ng, M. L. (2004). Interaction of West Nile virus with αvβ3 integrin mediates virus entry into cells. Journal of Biological Chemistry, 279(52), 54533-54541.
7. Dai, L., Song, J., Lu, X., Deng, Y. Q., Musyoki, A. M., Cheng, H., & Xiao, H. (2016). Structures of the Zika virus envelope protein and its complex with a flavivirus broadly protective antibody. Cell host & microbe, 19(5), 696-704.
8. Davis, C. W., Nguyen, H. Y., Hanna, S. L., Sánchez, M. D., Doms, R. W., & Pierson, T. C. (2006). West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. Journal of virology, 80(3), 1290-1301.
9. Dermime, S., Gilham, D. E., Shaw, D. M., Davidson, E. J., Meziane, E. K., Armstrong, A., & Stern, P. L. (2004). Vaccine and antibody-directed T cell tumour immunotherapy. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1704(1), 11-35.
10. Dhiman G, Lohia N, Jain S and Baranwal M. Metadherin peptides containing CD4þ and CD8þ T cell epitopes as a therapeutic vaccine candidate against cancer. Microbiol Immunol . 2016;60:646–652.
11. Diamond, M. S., Shrestha, B., Marri, A., Mahan, D., & Engle, M. (2003). B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. Journal of virology, 77(4), 2578-2586.

43

12. Dick, G. W. A., Kitchen, S. F., & Haddow, A. J. (1952). Zika virus (I). Isolations and serological specificity. Transactions of the Royal Society of Tropical Medicine and Hygiene, 46(5), 509-520.
13. Duffy, M. R., Chen, T. H., Hancock, W. T., Powers, A. M., Kool, J. L., Lanciotti, R. S., & Guillaumot, L. (2009). Zika virus outbreak on Yap Island, federated states of Micronesia. New England Journal of Medicine, 360(24), 2536-2543.
14. Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC bioinformatics, 5(1), 113.
15. Flower, D. R. (2013). Designing immunogenic peptides. Nature chemical biology, 9(12), 749.
16. Gollins, S. W., & Porterfield, J. S. (1984). Flavivirus infection enhancement in macrophages: radioactive and biological studies on the effect of antibody on viral fate. Journal of general virology, 65(8), 1261-1272.
17. Hailemichael, Y., & Overwijk, W. W. (2013). Peptide-based anticancer vaccines: The making and unmaking of a T-cell graveyard. Oncoimmunology, 2(7), e24743.
18. Hanna, S. L., Pierson, T. C., Sanchez, M. D., Ahmed, A. A., Murtadha, M. M., & Doms, R. W. (2005). N-linked glycosylation of west nile virus envelope proteins influences particle assembly and infectivity. Journal of virology, 79(21), 13262-13274.
19. Heinz, F. X., & Stiasny, K. (2012). Flaviviruses and flavivirus vaccines. Vaccine, 30(29), 4301-4306.
20. http://4.bp.blogspot.com/-kZBmRrrM3Lg/ThdtkKs7dAI/AAAAAAAAHwc/28bIKqbNJ-A/s400/flavivirus+life+cycle.jpg
21. http://jvi.asm.org/content/80/22/11000/F1.small.gif
22. http://jvi.asm.org/content/86/4/2337/F1.large.jpg
23. http://slideplayer.com/10205772/34/images/6/Systematic+Classification+of+Zika+Virus.jpg
24. https://talk.ictvonline.org/cfs-file/ key/communityserver-wikis-components-files/00-00-00-00-49/OPSR_5F00_Fig_5F00_2.v1.png
25. https://www.creative-diagnostics.com/images/Dengue%20Virus.png.
26. Ilekis, J. V., Reddy, U. M., & Roberts, J. M. (2007). Preeclampsia—a pressing problem: an executive summary of a National Institute of Child Health and Human Development workshop. Reproductive Sciences, 14(6), 508-523.

27. Ishak, R., Tovey, D. G., & Howard, C. R. (1988). Morphogenesis of yellow fever virus 17D in infected cell cultures. Journal of general virology, 69(2), 325-335.
28. Kaufmann, B., & Rossmann, M. G. (2011). Molecular mechanisms involved in the early steps of flavivirus cell entry. Microbes and infection, 13(1), 1-9.
29. Krek, A., Grün, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., & Rajewsky, N. (2005). Combinatorial microRNA target predictions. Nature genetics, 37(5), 495.
30. Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., & Baker, T. S. (2002). Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell, 108(5), 717-725.
31. Larocca, R. A., Abbink, P., Peron, J. P. S., Paolo, M. D. A., Iampietro, M. J., Badamchi-Zadeh, A., & Li, Z. (2016). Vaccine protection against Zika virus from Brazil. Nature, 536(7617), 474-478.
32. Lee, E., Hall, R. A., & Lobigs, M. (2004). Common E protein determinants for attenuation of glycosaminoglycan-binding variants of Japanese encephalitis and West Nile viruses. Journal of virology, 78(15), 8271-8280.
33. Leyssen, P., De Clercq, E., & Neyts, J. (2000). Perspectives for the Treatment of Infections withFlaviviridae. Clinical microbiology reviews, 13(1), 67-82.
34. Lindenbach, B. D., & Rice, C. M. (2003). Molecular biology of flaviviruses. Advances in virus research, 59, 23-62.
35. Lobigs, M., & Diamond, M. S. (2012). Feasibility of cross-protective vaccination against flaviviruses of the Japanese encephalitis serocomplex. Expert review of vaccines, 11(2), 177-187.
36. Lohia, N., & Baranwal, M. (2015). Identification of conserved peptides comprising multiple T cell epitopes of Matrix 1 protein in H1N1 influenza virus. Viral immunology, 28(10), 570-579.
37. Lohia, N., & Baranwal, M. (2017). Immune responses to highly conserved influenza A virus matrix 1 peptides. Microbiology and immunology, 61(6), 225-231.
38. Lorenz, I. C., Allison, S. L., Heinz, F. X., & Helenius, A. (2002). Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. Journal of virology, 76(11), 5480-5491.
39. Macdonald, J., Tonry, J., Hall, R. A., Williams, B., Palacios, G., Ashok, M. S., & Lipkin, W. I. (2005). NS1 protein secretion during the acute phase of West Nile virus infection. Journal of virology, 79(22), 13924-13933.

40. Mandl, C. W., Guirakhoo, F. A. R. S. H. A. D., Holzmann, H. E. I. D. E. M. A. R. I. E., Heinz, F. X., & Kunz, C. H. R. I. S. T. I. A. N. (1989). Antigenic structure of the flavivirus envelope protein E at the molecular level, using tick-borne encephalitis virus as a model. Journal of virology, 63(2), 564-571.
41. Markoff, L., Falgout, B., & Chang, A. (1997). A conserved internal hydrophobic domain mediates the stable membrane integration of the dengue virus capsid protein. Virology, 233(1), 105-117.
42. Meloen, R. H., Langeveld, J. P., Schaaper, W. M., & Slootstra, J. W. (2001). Synthetic peptide vaccines: unexpected fulfillment of discarded hope?. Biologicals, 29(3-4), 233-236.
43. Modis, Y. et al. (2004) Structure of the dengue virus envelope protein after membrane fusion. Nature 427, 313–319
44. Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. Nature, 427(6972), 313.
45. Mukhopadhyay, S., Kim, B. S., Chipman, P. R., Rossmann, M. G., & Kuhn, R. J. (2003). Structure of west nile virus. Science, 302(5643), 248-248.
46. Negida, A. I. A. A., & Ahmed, H. (2007). An updated review of Zika virus. proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), 3, 9.
47. Nybakken, G. E., Nelson, C. A., Chen, B. R., Diamond, M. S., & Fremont, D. H. (2006). Crystal structure of the West Nile virus envelope glycoprotein. Journal of virology, 80(23), 11467-11474.
48. Patronov, A., & Doytchinova, I. (2013). T-cell epitope vaccine design by immunoinformatics. Open biology, 3(1), 120139.
49. Petersen, L. R., & Roehrig, J. T. (2001). West Nile virus: a reemerging global pathogen. Emerging infectious diseases, 7(4), 611.
50. Pierson, T. C., Fremont, D. H., Kuhn, R. J., & Diamond, M. S. (2008). Structural insights into the mechanisms of antibody-mediated neutralization of flavivirus infection: implications for vaccine development. Cell host & microbe, 4(3), 229-238.
51. Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C., & Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. Nature, 375(6529), 291.
52. Roberts, Michelle. "Zika-linked condition: WHO declares global emergency". BBC News. Retrieved 1 February 2016.

53. Rodenhuis-Zybert, I. A., van der Schaar, H. M., da Silva Voorham, J. M., van der Ende-Metselaar, H., Lei, H. Y., Wilschut, J., & Smit, J. M. (2010). Immature dengue virus: a veiled pathogen?. PLoS pathogens, 6(1), e1000718.
54. Roehrig, J. T., Johnson, A. J., Hunt, A. R., Bolin, R. A., & Chu, M. C. (1990). Antibodies to dengue 2 virus E-glycoprotein synthetic peptides identify antigenic conformation. Virology, 177(2), 668-675.
55. Shafique, S. (2017). Envelope protein structure of Zika virus-A target for vaccine development and therapeutics. Annals of Clinical Trials and Vaccines Research, 1(1).
56. Shrestha, B., & Diamond, M. S. (2004). Role of CD8+ T cells in control of West Nile virus infection. Journal of virology, 78(15), 8312-8321.
57. Sikka, V., Chattu, V. K., Popli, R. K., Galwankar, S. C., Kelkar, D., Sawicki, S. G., & Papadimos, T. J. (2016). The emergence of Zika virus as a global health security threat: a review and a consensus statement of the INDUSEM Joint Working Group (JWG). Journal of global infectious diseases, 8(1), 3.
58. Stadler, K., Allison, S. L., Schalich, J., & Heinz, F. X. (1997). Proteolytic activation of tick-borne encephalitis virus by furin. Journal of virology, 71(11), 8475-8481.
59. Stiasny, K., & Heinz, F. X. (2006). Flavivirus membrane fusion. Journal of general virology, 87(10), 2755-2766.
60. Stiasny, K., Kössl, C., Lepault, J., Rey, F. A., & Heinz, F. X. (2007). Characterization of a structural intermediate of flavivirus membrane fusion. PLoS pathogens, 3(2), e20.
61. Szretter, K. J., Daniels, B. P., Cho, H., Gainey, M. D., Yokoyama, W. M., Gale Jr, M., & Diamond, M. S. (2012). 2′-O methylation of the viral mRNA cap by West Nile virus evades ifit1-dependent and-independent mechanisms of host restriction in vivo. PLoS pathogens, 8(5), e1002698.
62. Tassaneetrithep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., & Steinman, R. M. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. Journal of Experimental Medicine, 197(7), 823-829.
63. Van der Schaar, H. M., Rust, M. J., Waarts, B. L., van der Ende-Metselaar, H., Kuhn, R. J., Wilschut, J., & Smit, J. M. (2007). Characterization of the early events in dengue virus cell entry by biochemical assays and single-virus tracking. Journal of virology, 81(21), 12019-12028.

64. Villar, L., Dayan, G. H., Arredondo-García, J. L., Rivera, D. M., Cunha, R., Deseda, C., & Rey, L. C. (2015). Efficacy of a tetravalent dengue vaccine in children in Latin America. New England Journal of Medicine, 372(2), 113-123.
65. Xu, X., Vaughan, K., Weiskopf, D., Grifoni, A., Diamond, M. S., Sette, A., & Peters, B. (2016). Identifying candidate targets of immune responses in Zika virus based on homology to epitopes in other flavivirus species. PLoS currents, 8.
66. Yu, I. M., Zhang, W., Holdaway, H. A., Li, L., Kostyuchenko, V. A., Chipman, P. R., & Chen, J. (2008). Structure of the immature dengue virus at low pH primes proteolytic maturation. Science, 319(5871), 1834-1837.
67. Zhang, Y., Corver, J., Chipman, P. R., Zhang, W., Pletnev, S. V., Sedlak, D., & Rossmann, M. G. (2003). Structures of immature flavivirus particles. The EMBO journal, 22(11), 2604-2613.
68. Zhang, Y., Zhang, W., Ogata, S., Clements, D., Strauss, J. H., Baker, T. S., & Rossmann, M. G. (2004). Conformational changes of the flavivirus E glycoprotein. Structure, 12(9), 1607-1618.
69. Zhou, Y., Ray, D., Zhao, Y., Dong, H., Ren, S., Li, Z., & Li, H. (2007). Structure and function of flavivirus NS5 methyltransferase. Journal of virology, 81(8), 3891-3903.