Exploring zebrafish as an immunological model organism for investigating celiac disease and identification of viral MHC-I specific peptides

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Certificate of Examination

This is to certify that the dissertation titled ***"Exploring zebrafish as an immunological model organism for investigating celiac disease and identification of viral MHC-I specific peptides"*** submitted by **Mr. Ankit Verma** (Reg. No. MP13002) for the partial full- filment of MS degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rajesh Ramachandran Dr. Shashi Bhusan Pandit Dr. Sharvan Sehrawat

(Supervisor)

Dated: 22th April, 2016

Declaration

The work presented in this dissertation has been carried out by me under the guidance of **Dr. Sharvan Sehrawat** at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Mr. Ankit Verma

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In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sharvan Sehrawat

(Supervisor)

Acknowledgement

First and foremost I would like to express my deep sense of gratitude to **Dr. Sharvan Sehrawat** for providing me opportunity to join his lab and providing me an opportunity to work in the lab. The unconditional freedom that he has given throughout my study helped me to work better and allowed me to work in different directions.

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This project has been an enriching experience and has helped me recognize the effort that is out behind any scientific endeavor.

Mr.Ankit Verma

(Reg.No.MP13002)

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List of Abbreviations

PCR	Polymerase chain reaction
MCT	Micro centrifuge tube
MaCT	Macro centrifuge tube
GuCl	Guanidinium chloride
SDS	Sodium dodecyl sulphate
PAGE	Poly-acrylamide gel electrophoresis
DNA	Deoxyribose nucleic acid
TAE	Tris-acetate-EDTA buffer
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween
CFSE	Carboxyfluorescein succinimidyl ester
CD	Celiac disease
DGP	Deaminated gliadin peptides
GLU	Glutenin
DTT	Dithiothreitol
APC	Antigen presenting cells
MHC	Major histocompatibility complex
ER	Endoplasmic reticulum
ABC	ATP binding cassette
CHIKV	Chikungunya virus
DENV	Dengue virus
ANN	Artificial neural network
ACE	Atomic contact energy

Chapter-I

1.1 INTRODUCTION

It was not very long ago when you could either be vegetarian or non-vegetarian but now you can also be non-glutenarian (who do not eat gluten). Gluten is correlated with three conditions in human viz. **celiac disease (CD** an autoimmune disorder**), non-coeliac gluten sensitivity** and **wheat allergy**. Gluten is a protein found in endosperm of wheat and wheat related grains. It is composed of two types of protein named gliadin (alcohol soluble) and glutenin (dilute acid soluble)[1]. Gliadin derived peptides are responsible for symptoms like diarrhea, intestinal cramps, anemia and severe abdominal pain[2] in susceptible individuals. It has been more than several decades since celiac diseases were described but unfortunately even today there is no drug available in the market for managing celiac disease in patients. The only treatment available for CD is not to eat gluten lifelong. This problem prevails mostly in western countries because of genetic susceptibility that can be ascribed to having a specific genetic makeup (HLA-DQ2/DQ8 haplotype)[3] which presents gliadin derived peptides to specific CD4 T cells. 1%-4% European and American population is suffering from gluten related diseases.

A Dutch pediatrician (W. K. Dicke) made an incredible observation during World War II in children having symptoms of diarrhea, intestinal cramps, and severe abdominal pain. Thus he observed that mortality rate decreased remarkably during World War II and then again increased to the same level in post war times. A comparison of diet pre during and post war times revealed that a significant correlation existed with wheat availability. Then those kids were removed from wheat consumption and fed with banana exclusively for several months. Because of this change is feeding habit from wheat to banana those were named as "banana kids". Surprisingly the death rate came down to zero. After several months of exclusive banana diet they were eventually and slowly restored to normal diet[4]. When wheat was

reintroduced in six children the specific symptoms reappeared. This provided an explanation that indeed wheat consumption was responsible for the symptoms.

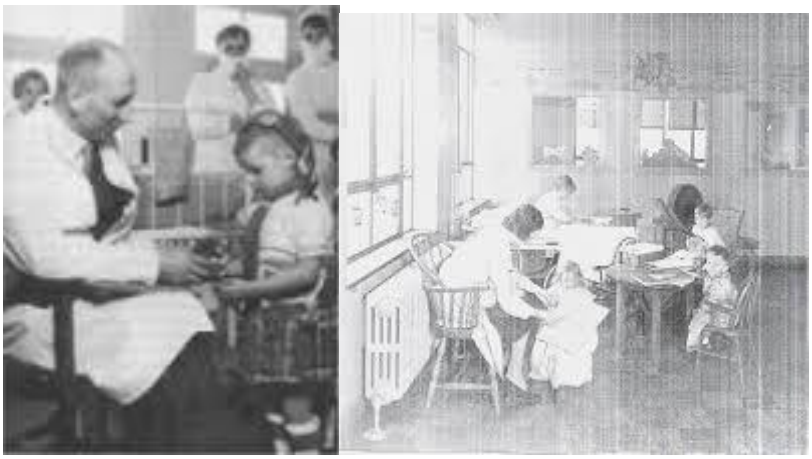


Fig 1.1 Gut 1993; **34:1473-**1475 doi:10.1136/gut.34.11.1473

Ironically, even after 75 years of its discovery the same line of intervention is still being followed that was described by W. K. Dicke. Thus, no single drug to ameliorate this condition is available in market. Since the first description of its etiology we have come a long way in understanding the pathogenesis of disease and it is well established now that this disease is not a food allergy but an autoimmune disease. **Tissue transglutaminase**[5] is already been identified as an autoantigen for instigating the disease. Antibodies that specifically bind to tissue transglutaminase enzyme are used as a diagnostic method to identify the disease. As wheat is an integral part of every civilization, it is expected that patients of celiac disease would be present throughout the world.

1.1.1 Symptoms and Diagnosis

Phenotypic symptoms of any disease have minimum two components viz. genetic factor, environmental factor however as has been recently appreciated microbiome may constitute a third factor. For CD the environmental factor is **gluten** and the most of the CD patients share a genetic component, which is MHC haplotype HLA DQ2/DQ8. Autoimmune disease has a huge penetrance of a gene or set of genes therefore; CD itself could potentially serve as a

model disease for understanding the pathophysiology of autoimmune diseases and provides an opportunity to devise strategies to deal with this disease specifically and other autoimmune diseases generally. The correlation of having gluten in diet and development of a disease in genetically susceptible individuals is so strong that disease can be made better or even reverted back in these patients upon the removal of gluten. This is because neither an atrophied intestinal nor elevated levels of anti-tissue transglutaminase antibodies persist upon removal of gluten from the diet. The gastrointestinal symptoms exhibited by genetically susceptible individuals those on gluten diet include: Chronic and recurrent diarrhea, abdominal distension, anorexia, failure to thrive and weight loss, abdominal pain, vomiting, constipation and irritability. Earlier CD was considered as a gut related disease while systemic symptoms are well evident as a result and these include: dermatitis herpetiformis, dental enamel hypoplasia of permanent teeth, osteopenia or osteoporosis, short stature, delayed puberty, iron-deficient anemia, resistant to oral iron, hepatitis, arthritis, epilepsy with occipital calcification.

Several tests are used to deduce the presence of CD. Serological tests are the front-line information required to make a diagnosis of coeliac disease. Anti-endomysial antibodies of the immunoglobulin A (IgA) type may detect coeliac disease with a sensitivity and specificity of 90% and 99%, respectively. Serology for anti-tTG antibodies was initially reported to have a higher sensitivity (99%) and specificity (>90%) for identifying coeliac disease.

1.1.2 Epidemiology

Latest survey indicates that celiac disease prevails at much more frequency as it was thought earlier. In United States of America approximately 1% of the current population was found to exhibit CD symptoms. Sweden and Finland fall amongst those countries which have highest prevalence of CD which is up to 2%-3%[7]. One interesting elucidation during some recent studies is that Germany carries only 0.2% CD patient which is huge change in spite of almost same distribution of causal factors which include the amount of gluten intake and

genetic haplotype. This gives rise to a possibility that there some causal factor waiting to be discovered.

1.1.3 Gluten

Gluten protein is very elastic in nature. It may sound a little bit ordinary but because of its elastic nature the tri-composition of water yeast and wheat flour makes a very beautiful thing called bread. If we eat a protein in order to make full use of it, we have to digest the protein into its basic components (amino-acid). Since gluten is so elastic and so unique in its composition (very high content of glutamine 40% and proline 14%)[8]. it cannot be digested by our gut system. It makes a cocktail of peptides in our gut as our proteases such as pepsin and chymotrypsin are unable to digest it completely. Human species cannot dismantle gluten completely. When immune system tries to fight a foreign entity, a bidirectional damage happens which is known as inflammation. There are two motifs (blue) that are present in gliadin protein which are responsible for leaky gut by the action of Zonulin at tight junction. Zonulin is protein, which is released by intestinal cells and it loses the tight junction in its near vicinity. The motif in light green has immunomodulatory function while the motif in dark green induces CXCR3 dependent IL8 release[8].

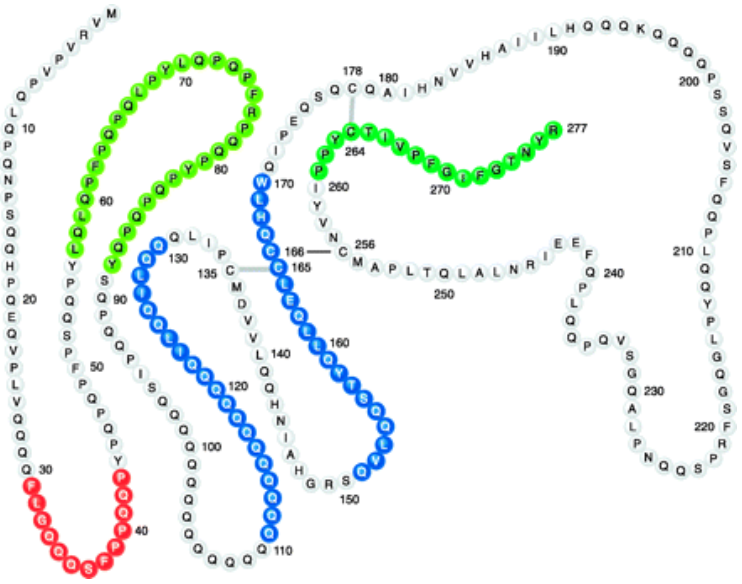


Fig 1.2 Gliadin motifs. Mapping of α-gliadin motifs exerting cytotoxic activity (red), immunomodulatory activity (light green), zonulin release and gut-permeating activity (blue), and CXCR3-IL-8 release in CD patients (dark green).

1.1.4 Immunological recognition of gluten in causation of disease

Gluten is the proteinaceous component of wheat, which is responsible for CD. It is composed of two types of proteins gliadin, an alcohol soluble component and Glutenin, a mild acid soluble component. Gliadin contains many glutamine and proline amino-acids which make it protease resistant and hence it is not digested completely in gut and a cocktail of larger peptides are generated. Some of these are presented by MHC class II haplotypes HLA-DQ2/DQ8 to help induce $CD4^+$ T cell response against some distinct gliadin peptides. Gliadin, which is the main culprit protein also plays some dramatic roles such as there are many different forms are available such alpha, beta, gamma and omega. Mouse are more responsive to alpha gliadin, all the studies done in mice are in context of alpha gliadin while some recent papers shows the involvement of gamma gliadin in humans. This could be a turning point in CD research. Gliadin can also form dimer, trimer and metameres that could potentially induce specific B cell response and hence the involvement of humoral immunity

in causation. In addition the role of CD8 T cells in the causation of CD has been ill explored and could provide further insights into the pathophysiology of CD.

1.1.5 Known immunological mechanisms that set the stage of CD causation

Human gut proteases are unable to digest gliadin completely; consequently a cocktail of peptides is generated. These peptides bind to **CXCR3**[9] receptor (on intestinal cells) and induce the secretion of **Zonulin**, which is capable of modulating the permeability of tight junctions between intestinal cells. The leaky gut allows peptides to enter into the system, now these peptides further binds to extracellular **tissue transglutaminase enzyme (tTG).** This association of tTG and gliadin peptide acts an altered self-antigen, which induces a cell-mediated immune response. Production of proinflammatory mediator disrupts intestinal wall and it also leads to other immunopathological responses.

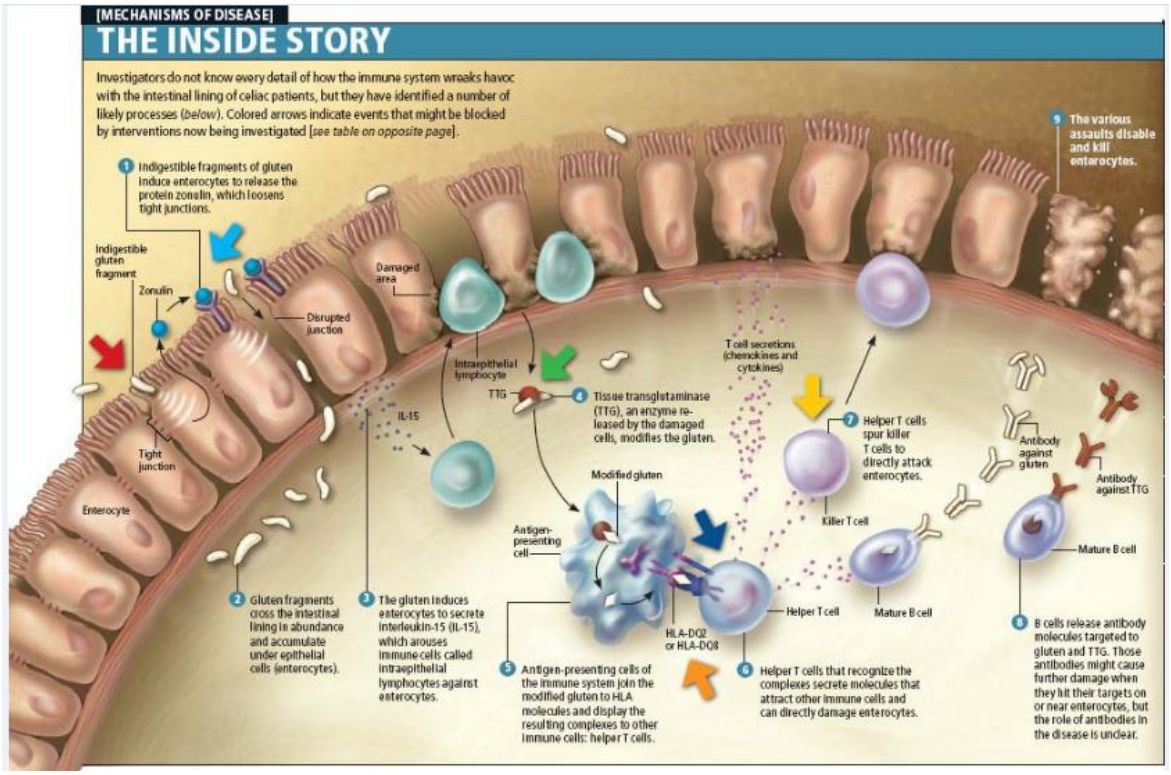


Fig1.3 Mechanism of celiac disease (Please refer to under given URL for clear image) http://www.nature.com/scientificamerican/journal/v301/n2/full/scientificamerican0809-54.html

1.1.6 Diagnosis of gluten

The main accomplishment that has happened in gluten related disorder is that we have increased the sensitivity of different serological markers.

In-spite of having some reliable tools we need to work on many thing and future direction for better tools. There is an algorithm for these test by which we could know indeed the person has celiac disease. We also have to eliminate intestinal biopsy from the picture as it is costly and just a small piece of intestine may lead you to some true negative results.

TEST	SENSITIVITY (RANGE)	SPECIFICITY (RANGE)	COMMENTS
IgA-anti-tTG antibody	>95% (73.9-100)	>95 (77.8-100)	It is suggested as first level screening test
IgG anti-tTG antibody	widely variable (12.6-99.3)	widely variable (86.3-100)	It is useful in those patients in IgA level is low
IgA antiendomysial antibody	>90% (82.6-100)	>98.2% (94.7-100)	useful in patients with uncertain diagnosis
IgG DGP	>90%(80.1-98.6)	>90% (86.0-96.9)	useful in patients with IgA deficiency
HLA-DQ2 or HLA-DQ8	>91.0% (82.6-97.0)	>54% (12.0-68.0)	High negative predicted value

Reference: Fasano and Catassi NEJM 2012:367:2419-26

1.1.7 Zebrafish as an Immunological model system

Zebrafishes are among earliest organism as far as evolutionary perspective is considered who have both innate as well as adaptive immune system, which is same as mammal (innate and acquired). Zebrafish and human have approximately 75% similar genome. The most important feature of zebrafish is their being transparent up to early larval stage, which gives all sorts of live imaging techniques to work on transparent fish. Their transparency enables us to see immunity in action. A zebrafish on an average spawn more than 200 eggs per week. They are comparatively cheaper to house. In order to interrogate any biological issue, it is essential to alter one or more genes and zebrafish allows altering of genes either temporarily or permanently. Native place of zebrafish is tributaries of Ganges River in Bangladesh and India. They are very hardy animals and survive very well in

minimal conditions; this is the reason why zebrafish can flourish in any fish tank around the world. It has been used since 1970's for drug testing, regenerative and developmental studies but it has just started its race as an immunological model. In 2006 the zebrafish was used to study the development of lymphocytes.

1.2 Material Methods:

1.2.1 Gliadin Extraction

Gluten protein is elastic in nature. This property is utilized in making wheat breads. Owing to the elasticity and unique composition of gluten, it is not easily digested by proteases in gut. Therefore, it generates a cocktail of peptides in gut as our proteases such as pepsin and chymotrypsin are unable to digest it completely. Many different proteins are present in a seed of wheat and the relative concentration of these proteins varies with the varieties of wheat. These proteins are extracted on the basis of their solubility. A description of different main proteins that are found in wheat is as follows.

- Albumin: A protein which is soluble in water and some diluted aqueous buffers, it is a monomeric non-gluten protein and provides protection from pathogens.
- Globulin: Globulin is a protein which is soluble in dilute salt solutions and is a monomeric non-gluten protein which has important role in supplying food reserves to embryo during developmental stages.
- Gliadin: A protein which is soluble in diluted alcohol solution. A yield of 60% to 75% in alcohol extracted fraction. It is a seed storage protein. This is one of the proteins, which is responsible for elasticity of dough.
- Glutenin: A protein, which is soluble in dilute acetic acid and exists in polymeric forms. It is also a seed storage protein and it is responsible for elasticity of dough.

There are different methods available for extraction of gliadin protein. Six different methods were used in this study to establish which method was most efficient.

Method No. 1

Jacek Waga, Jerzy Zientarski, Pol. J. Food Nutr. Sci. 2007, Vol. 57, No. 1, pp. 91–96[10]

- 4 mg of wheat flour was mixed with 800mM NaCl (40ml) solution and was incubated for overnight at constant stirring.

- Next morning, it was centrifuged at 8,000 rpm and the supernatant was discarded which had albumin in it.
- 40ml of 60% ethanol was added in remaining pallet. It was vortexed for 5 minute and incubated at 4° Celsius overnight at constant stirring.
- Next day, the solution was centrifuged at 8,000 rpm and yellowish looking supernatant was collected in another tube.
- 5ml of supernatant was collected in another tube and 10 ml of absolute ethanol was added.
- Solution became whitish and turbid.
- This solution was then centrifuged at 11, 000 rpm for 15 minutes at 4°C.
- The supernatant was discarded and the pallet was dissolved in 1M guadinium chloride (GuCl).
- 20**µl** of protein solution was taken and it was run on 15% SDS Gel to check the gliadin extraction.

Method No. 2

Slađana Žilić , Miroljub Barać , Mirjana Pešić , Dejan Dodig Int. J. Mol. Sci. ***2011****, 12, 5878-5894*[11]

Albumin-Globulin extraction:

Wheat flour (0.5 g) was mixed with an aqueous solution of 0.5M NaCl (10mL) and stirring was done continuously for overnight at room temperature.

Solution was centrifuged at 10,000 rpm for 15 min at 4°C. Supernatant composed of albumin and globulin extracts was transferred to another conical flask and 0.5M NaCl (50 mL) was added. All content were vortexed with double distilled water (10 mL) for 1 min, than it was incubated for 5 min at room temperature and centrifuged again. The supernatant was then discarded. Additional washes reduce the effect of salt. Pallet was mixed with 70% aqueous ethanol (10 mL) for 30 min at 4 °C. The solution mixture was centrifuged for 15 min at 20,000 g. This step was repeated three times, the supernatant was transferred to the conical flask and 70% ethanol (50 mL) was added. Soluble glutenin extraction was done by adding 7ml of solution composed of isopropanol (50%) and dithiothreitol (1%) in pallet of above conical flask

Method 3:

DuPont F. M., Chan R. , Lopez R. , Vensel W. H. , J Agric Food Chem. 2005 Mar 9;53(5):1575-84

1g of flour was taken and mixed with 10 ml of 50% of isopropanol and it was then centrifuged at 4500g for 10 min at 4°C. The 1-propanol was allowed to evaporate and the pallet was added with 0.4 mL 50% 1-propanol, 25 mM DTT, 25 mM TRIS HCl, pH 8.0 per 100mg of original flour amount then it was incubated overnight in constant stirring at 4°C. Next morning the solution was centrifuged and the gliadin rich pallet was dried to obtain the gliadin.

Method 4:

M. G. SCANLON, P. K. W. NG, D. E. LAWLESS, and W. BUSHUK Cereal Chem. 67(4):395-399 [13]

Flour was homogenized and defatted in cold acetone then it was centrifuged at 8000rpm for 15 min at 4° C. Acetone was discarded and allowed to evaporate. The pallet was suspended in 0.5 M NaCl for 1hr at room temperature and this step was repeated 3 times. The pallet was re-suspended in 70% ethanol and the mixture was centrifuged at 4000rpm for 10 min at 20°C and then solution was lyophilized and used.

Method 5:

Aris Graveland, Pieter Bosveld, Willem J. Lichtendonk, Hans H. E. Moonen and Auke Scheepstra J. Sci. Food Agric. *1982,33, 1 117-1 128.* [14]

The wheat flour was taken and was dissolved in water and it was supplemented with 0.5% SDS in 1:20 ratio. It was centrifuged at 10, 000 rpm. Pallet had all the starch while the supernatant has protein portion. Supernatant was precipitated with 70% ethanol and it was centrifuged at 15000g. The supernatant was collected in a different flask and it was tested on SDS page.

1.2.2 SDS PAGE

Separating gel preparation:

- The casting frames were setup (two glass plates were placed with separators in the casting frame) on the casting stands.
- Solutions for gel were prepared with help of given table.

- **For a 10ml separating gel:**

Acylamide percentage	6%	8%	10%	12%	15%
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

Note: AP and TEMED must be added right before each use.

- The solution was swirled gently.
- The solution of gel was poured in-between two plates till 65% level.
- Top of separating gel should be even, so 1ml of isopropanol was added.
- Gel was allowed to solidify.

Isopropanol was removed before pouring stacking gel

Stacking gel:

- The solution for gel prepared with the help of given table.
  - **For a 5 ml stacking gel:**

H_2O	2.975 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

- Comb was inserted without tapering any bubble in-between.

- When the gel got solidified, the comb was removed gently.

Gel was allowed to solidify and comb was removed gently, casting frame along with plates and gel was placed in assembly and running buffer was poured.

Sample preparation:

- Samples were mixed with loading buffer.
- Samples were heated at 95 ° C for 10 min.
- Samples were loaded into the wells and it was made sure that there should not be any spillage of sample in another well.
- Assembly was started with 100V.

Compositions:

Staining solution (100ml)	
CBB	0.1g
Water	45ml
Methanol	45ml
Acetic acid	10ml

De-staining solution (100ml)	
Water	45ml
Methanol	45ml
Acetic acid	10ml

10X SDS PAGE Reservoir Buffer (500ml pH 8.3)	
25nM Tris Base	15.15g
192mM Glycine	72.05g
0.1% SDS	5g

Further level of purification was achieved with Size exclusion gel chromatography.

1.2.3 Size exclusion chromatography

- System was washed with water.
- It was equilibrated with 10M PBS buffer and then sample was injected.
- After 43 ml of void volume fraction collection was started (2ml each).
- The UV peak increased sharply it was monitored closely.
- Different were eluted and pooled together once peaks stopped to come.

Histochemical analysis of zebrafish intestine:

To deduce gliadin response in zebrafish gut. Fishes that were fed with gliadin at for different time point were sacrificed along with control fishes (protein diet but not gliadin). At the end of experiment, animals were sacrificed and their intestines were isolated. Same specific portion from each fish was isolated.

Intestinal Tissue fixation:

- Intestinal tissue was taken and kept in a micro centrifuge tube (MCT) containing 600µL of 4% PFA in 1X phosphate buffer.
- Mixing was done on rotator for two hours at RT.
- Previous solution was pipetted out and 600µL of 5% sucrose was added in MCT, it was mixed on rotator for 45 min at RT.
- Previous solution was pipetted out and 400µL of 5% and 200µL of 50% sucrose was added in MCT, it was mixed on rotator for 45 min at RT.
- Previous solution was pipetted out and 300µL of 5% and 300µL of 50% sucrose was added in MCT, it was mixed on rotator for 45 min at RT.
- Previous solution was pipetted out and 200µL of 5% and 400µL of 50% sucrose was added in MCT, it was mixed on rotator for 45 min at RT.

- Previous solution was pipetted out and 600µL of 20% sucrose was added in MCT, it was mixed on rotator for 45 min at RT.
- MCT was kept at 4^oC for 45 min.
- 600µL of OCT was added in it and mixed on rotator for 30 min.
- Tissue was fixed in OCT at 80^oC.

Composition of solutions used for tissue fixation:

1) 4% PFA in 1X Phosphate buffer: 2g PFA and 5mL of 10X phosphate buffer
Volume makeup till 50mL using autoclaved deionized H_2O (stored at -20^oC)
2) 5% sucrose: 2.5g sucrose dissolved in 50mL of autoclaved deionized H_2O and stored at 4^oC
3) 20% sucrose: 10g sucrose dissolved in 50mL of autoclaved deionized H_2O (stored at 4^oC)
Fixed tissue was sectioned using cryosectioner and stored at -20^oC,
OCT (Optimal cutting temperature compound) was used to make thin sections of intestine. Sections were then **stained with hematoxylin and eosin** to measure if there is differential infiltration of cells. Furthermore, sections were analyzed for villous atrophy.

Measuring the proliferation of zebrafish splenocyte in presence of gliadin:

Spleen from different groups of zebrafishes was taken out and a single cell suspension was made. Splenocytes thus obtained were cultured in media (10% FBS+ RPMI) alone or medium having crude gliadin (containing all forms) and purified gliadin (alpha/beta/gamma). However in order to estimate the proliferation of splenocytes cells were labeled with CFSE. Cells were then analyzed after incubating for 72 hours using flow cytometry.

1.3 Results and discussion:

As mentioned above six different methods were adopted in order to find out the best method for gliadin extraction. The reason behind multiplicity in the extraction method is occurrence of different strains of wheat. Different methods are suggested for different strains.

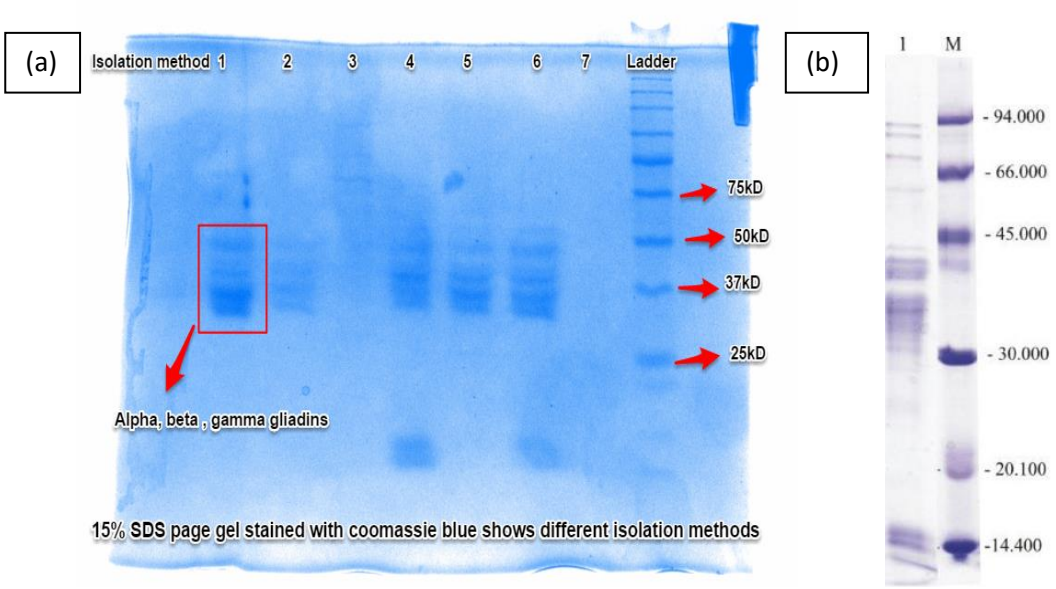


Fig 1.4 (a) Different lane showing different extraction method, Lane1- Method1, Lane2-diluted sample from Method1 {1:5}, Lane3- Method2, Lane4-Method, Lane5-Method4, Lane5-method5,Lane6-Method5 (b) Image taken from Int. J. Mol. Sci. 2011, 12, 5878-5894;for reference.

Lane 4 and lane 6 exhibited two bands at around 14KD which corresponds to albumin. The presence of albumin eliminated the method 3 and 5. We could not find anything in lane 3 so that's why corresponding method was also eliminated. Lane 1 had the most intensed band and therefore method 1{refer to page no. 9} was selected.

Bradford Assay:

The concentration of different gliadin from different methods were determined.using Bradford assay using BSA as a standard.

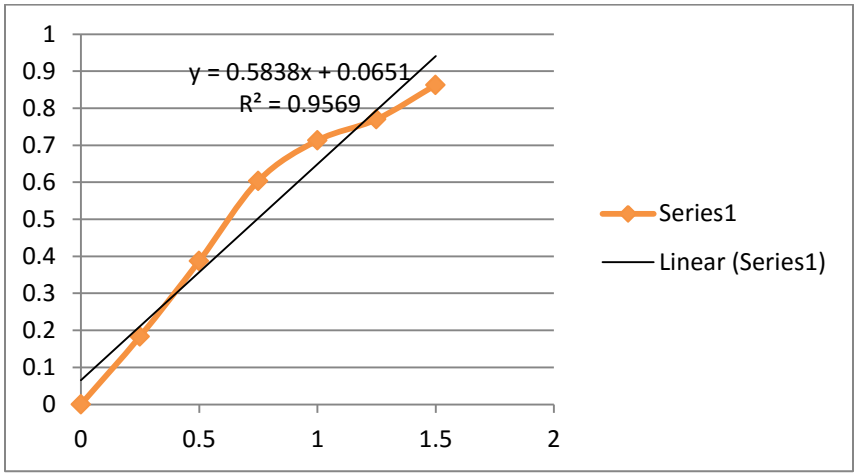


Fig1.5 Standered curve for BSA

BSA protein(mg/ml)	absorbance
0	0
0.25	0.183667
0.5	0.387334
0.75	0.603667
1	0.713
1.25	0.77
1.5	0.862667
Method 1	0.802
Method 2	0.0432
Method 3	0.6123
Method 4	0.68933
Method 5	0.734

1.3.1 Calculations:

Y=0.5838x+0.0651

Method1= 0.5838(0.802)+0.651

Method 1= 1.119mg/ml, Method 2= 0.67mg/ml, Method 3= 1.007

Method 4= 0.962mg/ml Method 5= 1.078mg/ml

All isolated proteins were subject to bradford test deduce their respective concentration. All protein came approximately 1mg/ml exept method 2.

Method 1 was choosen for isolation of gliadin protein because of the yield and purity and the sample was subjected to size exclusion chromatography. The motive behind doing size exclusion chromatography was to use purified protein fractions for further studies such as splenocyte proliferation assay or to feed zebrafish to check immunogenic response against gliadin.

1.3.2 Size exclusion chromatography:

Gliadins are found in mainly three forms alpha, beta, gamma gliadins. Their weight varries between 35KD to 44KD and relative quantity of alpha, beta, and gamma varies extinsively among different kind of strains. It was very difficut to predict exactly which elution correspond to which type of gliadin. Size exclusion chromatography helped us to achieve a level of purity not the identification of protein. Purified proteinproteins was part of bigger picture that if we get some immune response against gliadin mixtue then the next question was which type of gliadin is responible for inducing immune response. Almost all studies are being done in mice where alpha gliadin mediated peptides generates immune response and In human it has been shown that gamma gliadin is main culprit, studies with purified protein will help us to elucidate which portion of gliadin inducing immune responsed in Zebrafish. If it is able to generate immune response against gamma gliadin then zebrafish can act as better model organism.

Different peaks corresponding single type of protein were eluted with the help of size exclusion chromatography. Here we obtained six different fraction of proteins. These protein

were eluted in mcrocentrifuge tubes. Fractions were very highly diluted. All diluted proteins were concentrated with the help of concentrator.

As the heaviest protein in gliadind is gamma gliadin and lightest is alpha gliadin so atleast we can speculate that the first peak corresponds to gamma gliadin and peak 6 corresponded to alpha gliadin.

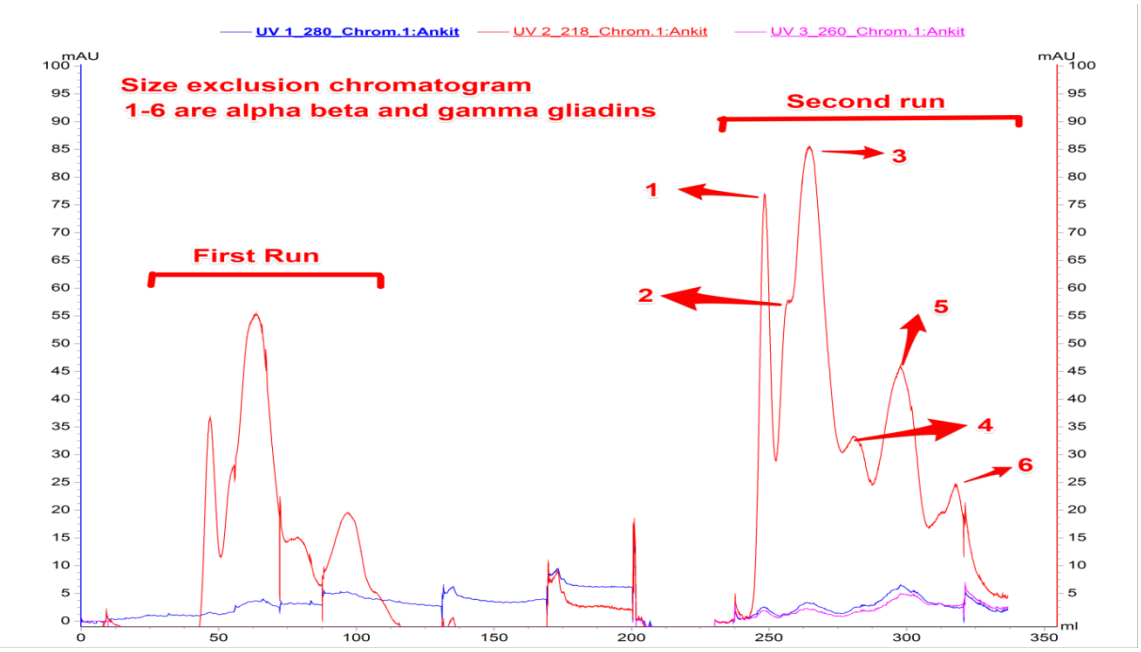


Fig 1.6 Size exclusion chromatogram shows two times run of extracted gliadin, different peaks corresponding to different type of gliadin.

1.3.3 Yield of polypeptides in fractionated gliadin:

The concentration of different types of gliadins were checked. Concentration was checked with the help of bradford assay.

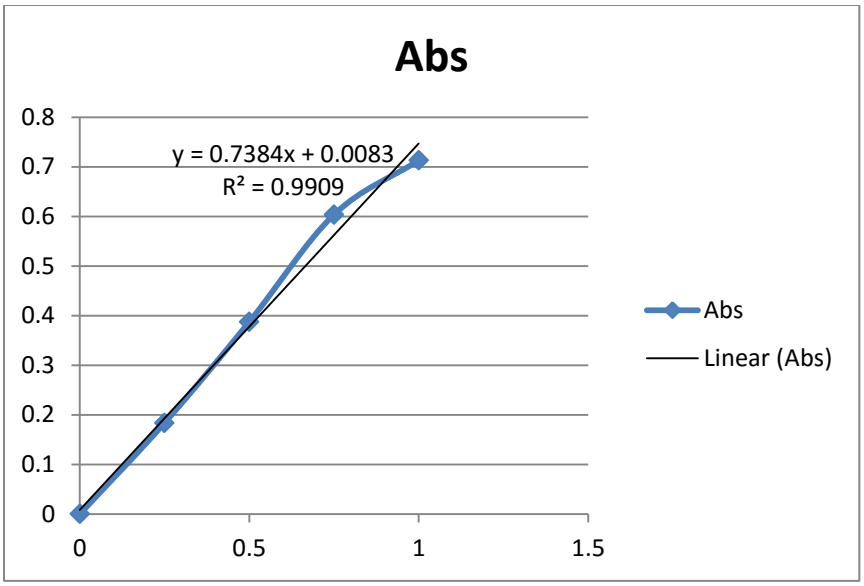


Fig1.7 Standered curve for BSA

BSA protein(mg/ml)	Absorbance
0	0
0.25	0.183667
0.5	0.387334
0.75	0.603667
1	0.713
1.25	0.77
1.5	0.862667
Peak 1	0.1023
Peak 2	0.0953
Peak 3	0.132
Peak 4	0.024
Peak 5	0.0290
Peak 6	0.01002

1.3.4 Calculations:

Y=0.7384x+0.0083

Peak 1= 0.08mg/ml Peak 2= 0.076mg/ml Peak 3= 0.103mg/ml

Peak 4= 0.026mg/ml Peak 5= 0.029mg/ml Peak 6= 0.015mg/ml

1.3.4 Histochemical analysis of zebrafish intestine:

Zebrafishes were divided into two groups. One subset was fed with gliadin only diet while another was on normal diet. Food was given 3 times a day. Same amount of food was given to both subset. Ater 2 weeks fishes were sacrified and intestinal tisssue was studies as given in material and method section.

1.3.5 Villous Atrophy and Cellular infiltration in Intestinal tissue:

Ptients with gliadin senssitivity tends to show villous atrophy and cellular infiltration in gut lining. In different groups of animals we did not observe any striking different in the histological sections either in the atrophy induced in villi or inflitration of inflammatory cells. There could be different explanations for these observations. The dose of gliadin that was fed may not be sufficient to induce any significant inflammation of intestine. The duration of treatment may not be enough to induce concievabe differences in histological observations. Zebrafish indeed may be not be susceptible genetically for gialdin induced immunoinflammatory lesions in the intestine.

Proliferation of spleen cells:

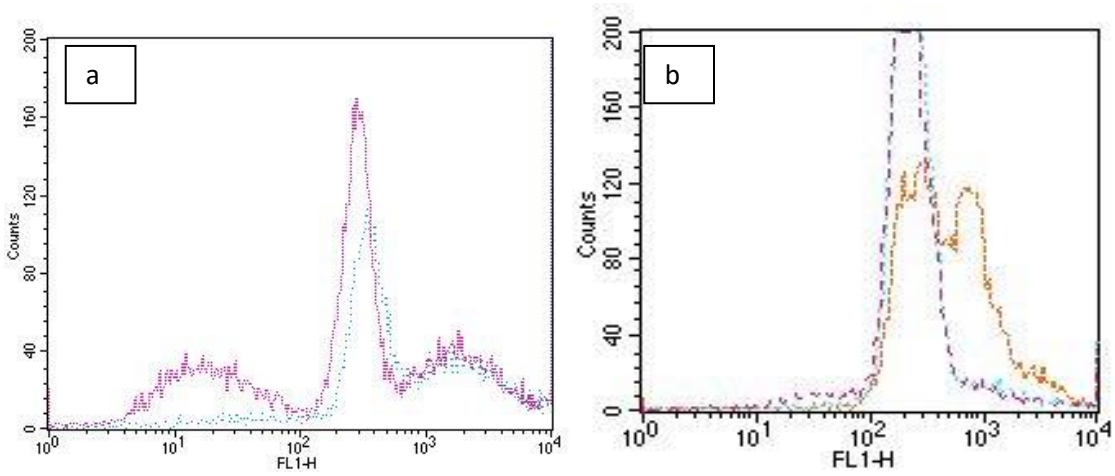
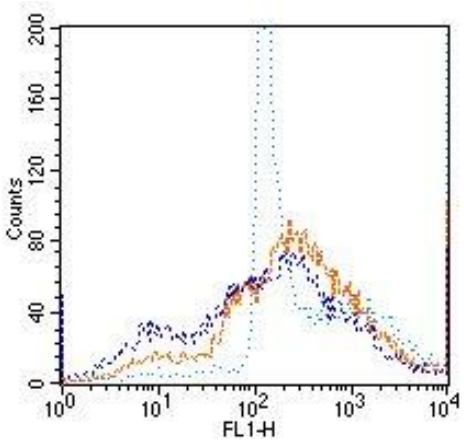


Fig.1.8 (a) showing the proliferation in splenocytes when it was incubated with gliadin in comparison to (b) when where it was incubated with PBS.

In order to investigate whether or not feeding of gliadin diet induces antigen specific cell proliferation, proliferation assays were performed. For this splenocytes isolated from the spleen samples of animals indifferent groups were pulsed in vitro with purified gliadin. As shown in Figure (1.8), there was enhanced proliferation of spleen cells isolated from gliadin treated group which suggest that indeed gliadin feading might have induced antigen specific cells systemically. However this trend was not consistent and in some animals we did not observe any differences in proliferation (fig 1.9). There can be different explanations for these results as described in the previous section. For concluding whether or not zebrafish is able to respond to gliadin diet and serve as a model for celiac disease further experimentation need to be performed. It could be that peptides derived form gliadin were not produced in sufficient quantity and thus being recognised as immunogenic. All these issues need to be investigated further.

Fig1.9 Zebrafish splenocyte incubated with gliadin (blue) without gliadin (orange), 0 time point stained cells (blue).

This was among those unexpected results which were found during the course of experiment.



1.3.6 Histopathological Analysis:

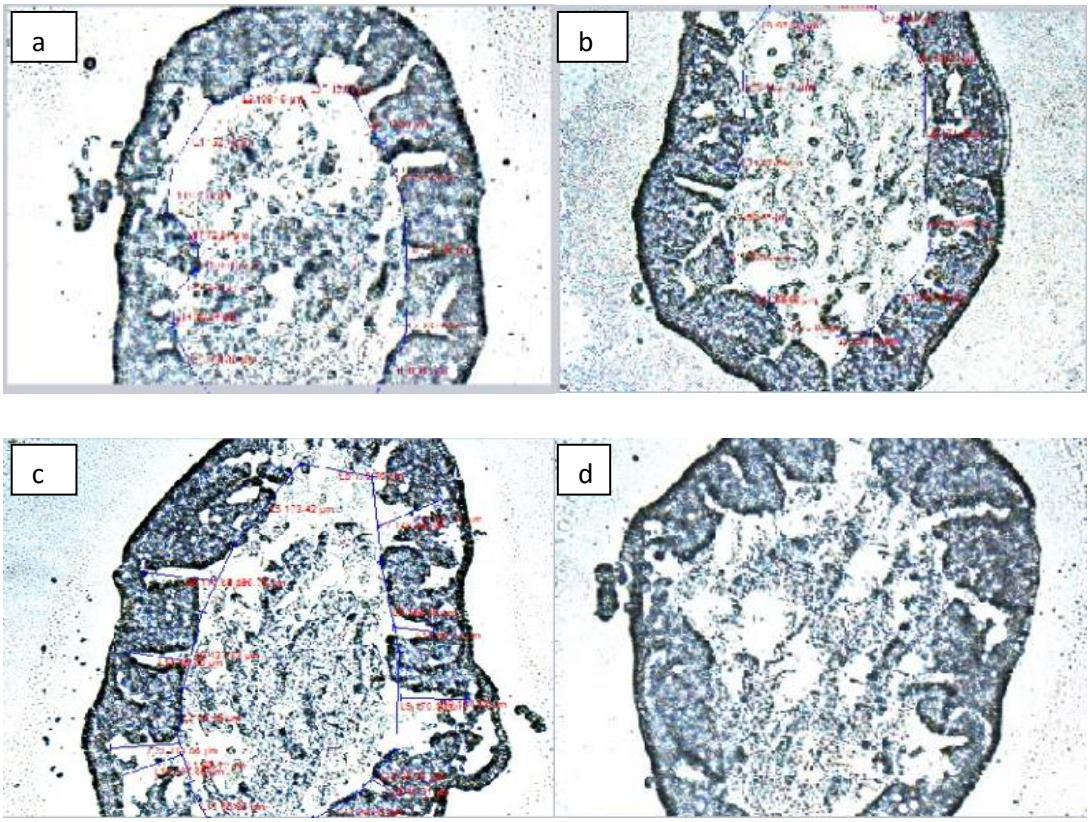


Fig. 1.10 a and b are control section of fish with normal diet. c and d are sections from fish with 2 week of gliadin diet. picture a,b,c,d shows no cellular infiltration or intestinal rugae and villi disruption.

1.4 Conclusion

The main motive behind this study was to acertain that which type of gliadin is responsible for immune reaction in zebrafish. Mice shows response against alpha gliadin while human shows responses against gamma gliadin. If zebrafish shows response aginst gamma gliadin then it can act as a better model for celiac disease.

In first picture it looks how could wheat, an integral part of human civilization be harmful for us but here is the key point, wheat could have been a part of human civilization but it is definitely not a part of human evolution. Wheat came up at the last second of our evolution. Initially we were hunter and gatherer we did not stay at a place for a long period. When humans learnt to do farming they stayed on one place and eat the food grown by them and wheat appeared to take its permanent place in human gut. Histopathological analysis of wheat did not show any villous atrophy or cellular infiltration. Histopathological slides show no response of gliadin on zebrafish intestine. Splenocyte proliferation assay shows some kind of positive indications regarding antigenic properties of gliadin. 1% of human population shows response against gliadin. We don’t know how much percent of zebrafish shows response against gliadin. In nutshell, we were not able to conclude whether zebrafish could serve as a model for celiac disease and further carefully planned studies may need to be performed.

Chapter II

2.1 INTRODUCTION

Dengue virus infections constantly pose a serious health threat in many parts of the world including India. India is among countries with most infected people in the world. Today almost 2.5 billion people, or 40% of the world's population, live in areas where dengue virus prevails. According to WHO approximately 60-100 million cases are there worldwide. A mosquito, *Aedes aegyptii*, transmits the virus. It is very common in warm and wet areas of world. In India only, more than ten lakh people are facing dengue infection and a large majority of them need hospitalization thereby it puts a very heavy burden on Indian economy. The virus of dengue is a member of *Flaviviridae* family and it has four different serotypes (DENV1, DENV2, DENV3 and DENV4). Primary infection is accompanied by symptoms like high fever, headaches, joint and muscle pain, vomiting, and rashes. But if a secondary infection occurs with a heterologous strain then those T cells or even B cell products that recognize epitopes with subtle variations are expanded enormously and lead to immunopathological manifestation in form of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The leakage of plasma after hemorrhagic fever and shock may lead to death.

2.1.1 Virology:

It is an RNA virus from the family *Flaviviridae*; genus *Flavivirus*. One of other example from same family is yellow fever virus. Since these viruses are transmitted by arthropods such as mosquitos and ticks they are also called arboviruses. Dengue can also spread through infected organ donation and infected blood transfusion.

2.1.2 Mechanism:

When a dengue virus-carrying mosquito bites a person, it enters inside the human system with mosquito's saliva. It reaches to blood and enter into white blood cell (WBC) produces different types of cytokines and proinflamatory mediators which are responsible for different symptoms during infection like fever, flu-like symptoms and severe pain in muscle and joints. The earliest detectable changes during the virus infection are decrease in WBC count and decrease in platelet count as well as increase in amount of ALT and AST. Laboratory tests used for detection include PCR amplification of viral genes or genetic fragments, antigens (NS1) detection with ELISA, virus specific IgG and IgM antibodies.

In the latest news Dengue virus vaccine entered phase 3 trial in Brazil, but it was not licensed in India. (https://www.nih.gov/news-events/news-releases/dengue-vaccine-enters-phase-3-trial-brazil)

Chikungunya virus (CHIKV) is carried by *Aedes: A. albopictus* and *A. aegypti*, Infection of virus leads to symptoms like fever and severe joint pain and rashes. Its incubation period may vary 2 to 22 days. CHIKV is member alpha virus genus. It is an RNA virus with positive sense single strand. This virus is also carried and transmitted by family Arthropoda that is why it also known as arbovirus. This virus can also transferred from mother to child but it is very highly unlikely. Virus enters in the body with mosquito bite and the virus is able to replicate inside the epithelial and endothelial cells, primary fibroblasts, and macrophages. Upon infection fibroblasts produces interferons. Diagnosis can be done with the help of PCR and serological detection of virus specific antibodies.

Immunological recognition of viruses:

Viruses are intracellular pathogens and, the viral genomic material starts to express and replicate itself inside the host cells. These proteins are ubiquitinized and thus tagged to be processed or cleaved predominantly through proteasome (fig 2.1). The proteasome chops the proteins and makes a cluster of peptides. Peptides, which are now able to align themselves with the transporter of antigen processing and presentation (TAP), can be internalized into ER. TAP is an ABC transporter present in the membrane of endoplasmic reticulum. However the role of other channels such as Sec61, derlins (derlin 1 and derlin II) has also been investigated recently, yet TAP remains the most characterized transporter system for shuttling peptides from cytosol to the ER. Now these peptides with the help of some chaperon proteins loads on to the Major histocompatibility complex MHC I molecule. Loading of peptides also starts the intracellular trafficking of MHC I molecule. Now APCs presents the MHC I molecule to CD8+ cytotoxic T-cell.

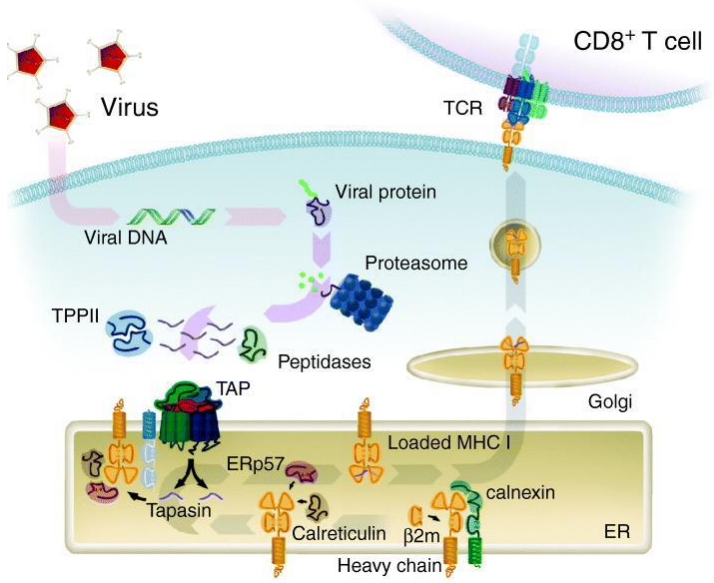


Fig. 2.1 Diagrammatic representation of MHC I antigen processing and presentation.
Ref. Groothuis et al., Immunological Reviews Vol. 207 (2005) 6

Specificity of TCR is solely dictated by the peptides presented in context of appropriate class of MHC-I. Therefore, the availability of MHC has facilitated the identification of antigen-specific T cells. The generation of Class I MHC tetramers is straightforward, and recent improvements involve the generation of Class I MHC tetramers loaded with conditional ligands such as photo cleavable peptides. In this procedure, tetramers are prepared with a photo cleavable derivative of a true Class I MHC ligand. Upon irradiation of these tetramers in the presence of synthetic peptides, the self-cleavage and exchange of the photo cleavable ligand for the peptide added during irradiation is highly efficient, and makes it possible to prepare, in a microtiter plate format, literally hundreds of distinct tetramers - using only small quantities of peptide- in a matter of hours. With this peptide exchange methodology, high throughput screens aimed at the detection of pathogen-specific T cells can be conducted in a genome-wide manner, certainly for pathogens whose genomes have modest coding capacity. Thus employing online peptide prediction programs, in vitro and in vivo assays and flow cytometry, epitope specific $CD8^+$ as well as $CD4^+$ T cells can be identified and enumerated in an unbiased manner. One of the advantages of this approach is the ability to identify, in humans or animals infected with the pathogen in question and through direct sampling of the responding primary T cells, a broader spectrum of $CD8^+$ T cells than would probably have been possible by relying on long term or cloned T cell lines.

Therefore, in this study we set to identify novel class I restricted immunogenic epitopes of DENV and CHIKV using a combination of bioinformatics tools and immunological assays.

Mice have six main haplotypes of MHC I molecules viz. H2-Kb, H2-Kd, H2-Db, H2-Dd, H2-Ld, H2-Kk. As C57Bl/6 and BALB/c mice are more commonly used for the studies involving pathogens owing to the availability of inbred strains, knockout or knock-in animals are necessary. Therefore, we took into account H2-Kb, H2-Db (most studied molecules of C57BL/6) and H2-Kd, H2-Ld (most studied MHC class I molecule of BALB/c strain of mice). Although we have taken into account all the protein but some protein were given more

preference as literature showed the presence of higher immunogenicity in some proteins which are E protein, partial [Dengue virus 1] GenBank: AFE84699.1, Chain A, Dengue Virus NS5, RNA Dependent RNA Polymerase (RDRP) of CHIKV and DENV.

2.2 Material and methods:

Sequence retrieval of amino acids:

Amino acid sequences of the structural polyproteins and non-structural polyproteins of CHIKV and DENV were retrieved from NCBI genbank and they were submitted to different online tools for T-cell epitope generation.

Online tools:

Different online and offline tools and software are available to predict the T-cell epitopes. These different online tools can generate a list of peptides on the basis of their respective criteria but since the presentation of antigen is not a single step process, it involves different steps such as protein cleavage, peptide transport; peptide loading etc., a single tool cannot predict a reliable list of peptide hits for t- cell epitopes prediction. These different tools were exploited and lists of hundreds of peptides were generated. These different lists were compared and those hits were funneled down which were common in at- least in two of them. Online tools used were netMHCpan(http://www.cbs.dtu.dk/services/NetMHCpan/) , IEDB recommended (http://tools.immuneepitope.org/mhci/), SMMPMBEC from Immune Epitope Database (http://tools.immuneepitope.org/mhci/), SYFPEITHI (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm), ANN (Artificial neural network).

Conservation analysis:

Since, we wanted to know that whether those peptides were specific to that particular virus. We did conservation analysis through protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSear7 | P a g e ch&LINK_LOC=blasthome) excluding the respective species from BLAST. This conservation analysis allowed us to further shortlist the peptides.

Molecular Docking:

Patchdock (http://bioinfo3d.cs.tau.ac.il/PatchDock/) was used to dock the pdb files of peptides made from PEP-FOLD (http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::rpbs.PEP-FOLD) to the pdb files of MHC I haplotypes. Since the X-ray crystallographic structures of MHC I molecules were with peptides at PDB (http://www.rcsb.org/pdb/home/home.do), same model without peptide was made with the help of Swiss model (http://swissmodel.expasy.org/interactive) and were used for docking.

2.2.1 RMA/S cell mediated peptide stabilization assay:

A peptide-binding assay with flow cytometry was performed as standardized in lab. RMA-S cell are grown at 37°C but rapid expression of MHC was observed at 26°C so RMA-S cells were cultured at 26°C for overnight and washed with serum-free medium. Then cells (1×10^5), in 200 μL of 10% RPMI, were added to tubes containing peptide 800μg/ml to 1μg/ml. These cells were incubated at 37°C overnight in incubator and next morning they were washed twice with cold (4°C) PBS. The cells were incubated for 30 minutes on ice with 50 μL of the antibody anti-mouse H2Kb (APC).The cells were then washed three times with PBS, after which fluorescence was measured with a flow cytometer.

2.2.2 Experimental validation of peptides from ELISA:

For validation we used two immunological techniques which were MHC class I stabilization assays on the surface of TAP deficient RMA/s cells, peptide exchange of MHC monomers using photo cleavable ligand followed by ELISA.

Following steps were performed for ELISA:

- ELISA Strips in 96 well plate were used and 5 μg/ml streptavidin in PB buffer was added (100μl per well).
- It was incubated overnight at 4°C. Plate was covered to avoid contamination and evaporation.
- Wells were washed 4 times, for 4 minute with 200μl wash buffer (0.05% tween in PBS).
- Blocking buffer (3% BSA in PBS) 200μl was added to each well.
- It was incubated for 2hr at room temperature. Plate was covered to avoid contamination and evaporation.
- Blocking buffer was removed and Washed 4 times, each for 4 minute with 200μl wash buffer (0.05% tween in PBS).
- 100μl MHC monomers were added which were taken from **EXCHANGE REACTION**.
- It was incubated for 2hr at room temperature. Plate was covered to avoid contamination and evaporation.
- Remaining buffer was removed and Washed 4 times, each for 4 minute with 200μl wash buffer (0.05% tween in PBS).
- 50μl of anti- β2microglobulin serum was added with 1:100 dilutions. (Different concentrations were tested and 1:100 was finalized)
- It was incubated for 2hr at room temperature. Plate was covered to avoid contamination and evaporation.

- Anti-mouse antibodies with enzyme alkaline phosphatase (1:10000 dilution)was added.
- It was incubated for 2hr at room temperature. Plate was covered to avoid contamination and evaporation.
- Remaining buffer was removed and Washed 4 times, each for 4 minute with 200µl wash buffer (0.05% tween in PBS).
- 200µl substrate (0.1M Glycine buffer+1mM ZnCl2 + 1mM MgCl2) was added.
- Development of the color was monitored by eye and absorbance was taken at 405nm.

EXCHANGE REACTION:

- 100µl of exchange buffer (20mM tris-HCl (pH7.0), 150mM NaCl) was supplemented with) 0.5µM MHC monomer and 50µM peptide.
- The plate was exposed to UV light (365nM) for 40 min, which should be 5-10cm away from plate.
- The plate was left at room temperature for one hour for exchange reaction to take place.

Compositions:

Wash buffer- 0.05% tween 20 in PBS

Exchange buffer- 20mM Tris-HCl (pH7.0), 150mM NaCl

Blocking buffer- 3% BSA in PBS

Phosphate buffer saline- PB (100mM) + NaCl (150mM)

Na_2HPO_4 = 8.340g, NaCl = 8.770g

KH_2PO_4 = 5.62g, makeup with water1000ml

Substrate buffer (p-nitro phenol) for alkaline phosphatase

P-nitrophenol = 1mg/ml

Glycine = 0.1mM MgCl2 = 1mM ZnCl2 = 1mM

2.3 Results and Discussion: List of predicted peptides

allele	seq_num	start	end	length	peptide con
H-2-Ld	2	39	47	9	IPTAMAFHL
H-2-Ld	9	51	59	9	SPCKIPFEI
H-2-Ld	26	50	58	9	FPQSNAPIM
H-2-Kb	1	51	59	9	VAFLRFLTI
H-2-Db	5	4	12	9	IGISNRDFV
H-2-Db	20	35	43	9	SLLKNDIPM
H-2-Db	34	32	40	9	YSQVNPITL
H-2-Db	48	11	19	9	LAVWNRVWI
H-2-Kb	3	23	30	8	ITYKCPLL
H-2-Kb	17	43	50	8	VAVSFVTL
H-2-Kb	32	15	23	9	SILLWYAQI
H-2-Db	18	45	53	9	VALLSQSTI
H-2-Db	31	16	24	9	NALDNLAVL
H-2-Kb	47	35	43	9	LMYFHRRDL
H-2-Kb	20	29	36	8	VSILASSL
H-2-Kb	4	29	37	9	TWILRHPGF
H-2-Kb	18	7	14	8	MGVTYLAL
H-2-Kb	32	16	23	8	ILLWYAQI
H-2-Kb	32	32	39	8	IILEFFLI
H-2-Kb	34	38	45	8	ITLTAALL
H-2-Kb	4	55	62	8	RALIFILL
H-2-Kb	17	43	51	9	VAVSFVTLI
H-2-Kb	33	46	54	9	VATTFVTPM
H-2-Kb	20	29	37	9	VSILASSLL
H-2-Kb	25	58	65	8	ATFTMRLL
H-2-Kb	32	32	40	9	IILEFFLIV
H-2-Kb	43	23	30	8	RAIWYMWL
H-2-Ld	35	19	27	9	EPIPYDPKF
H-2-Db	8	52	60	9	TALTGATEI
H-2-Db	41	32	40	9	MAMTDTTPF
H-2-Kb	37	58	65	8	WSYYCGGL
H-2-Kb	11	57	64	8	VSLVLVGV
H-2-Db	25	60	68	9	FTMRLLSPV
H-2-Db	35	13	21	9	ITVIDLEPI
H-2-Kb	43	33	40	8	RFLEFEAL
H-2-Kb	11	31	38	8	VSWTMKIL
H-2-Kb	23	61	68	8	VSLDFSPG
H-2-Kb	38	61	68	8	RTLRVLNL
H-2-Db	4	40	48	9	MAAILAYTI
H-2-Db	5	33	41	9	TMAKNKPTL
H-2-Kb	18	7	15	9	MGVTYLALL
H-2-Db	33	46	54	9	VATTFVTPM
H-2-Kb	33	48	55	8	TTFVTPML
H-2-Db	40	30	38	9	SEIPNLDII

H-2-Kb	23	61	69	9	VSLDFSPGT
H-2-Kb	1	9	16	8	RSTPFNML
H-2-Kb	11	41	48	8	VIITWIGM
H-2-Kb	36	10	17	8	IAVSMANI
H-2-Db	17	43	51	9	VAVSFVTLI
H-2-Db	21	15	23	9	SSPILSITI
H-2-Kb	45	61	69	9	VGRERLTRM
H-2-Kb	46	50	57	8	CSHHFHEL
H-2-Ld	8	32	40	9	NPHAKKQDV
H-2-Ld	41	26	34	9	VPMVTQMAM
H-2-Kb	10	15	23	9	VNIEAEPPF
H-2-Kb	36	15	23	9	ANIFRGSYL
H-2-Kb	1	48	56	9	MALVAFLRF
H-2-Kb	7	28	35	8	SSITEAEL
H-2-Db	26	17	25	9	ASIAARGYI
H-2-Kb	34	27	35	9	LAIGCYSQV
H-2-Kb	21	41	49	9	TILIRTGLL
H-2-Kb	39	61	69	9	SSVNMISRM
H-2-Ld	4	32	40	9	LRHPGFTIM
H-2-Db	19	39	47	9	LAAVSVSPL
H-2-Db	32	30	38	9	ASIILEFFL
H-2-Kb	38	18	25	8	STYGWNLV
H-2-Kb	43	47	55	9	HWFSRGNSL
H-2-Ld	1	27	35	9	QQLTKRFSL
H-2-Kb	4	58	65	8	IFILLTAV
H-2-Kb	17	34	41	8	VGTKHAIL
H-2-Kb	28	57	64	8	YIYMGEPL
H-2-Kb	16	39	46	8	RSCTLPPL
H-2-Kb	8	60	67	8	IQMSSGNL
H-2-Kb	11	31	39	9	VSWTMKILI
H-2-Kb	33	39	46	8	SAWTLYAV
H-2-Kb	36	26	33	8	AGLLFSIM
H-2-Kb	39	63	70	8	VNMISRML
H-2-Kb	46	50	58	9	CSHHFHELV
H-2-Kb	1	52	59	8	AFLRFLTI
H-2-Db	6	60	68	9	VQPENLEYT
H-2-Kb	17	48	55	8	VTLITGNM
H-2-Ld	29	15	23	9	TPEGIIPSM
H-2-Db	33	39	47	9	SAWTLYAVA
H-2-Db	35	51	59	9	WALCEALTL
H-2-Db	39	20	28	9	SVIEKMETL
H-2-Db	43	27	35	9	YMWLGARFL
H-2-Kb	20	30	37	8	SILASSLL
H-2-Db	25	22	30	9	VAAEMEEAL

Structural protein [Chikungunya virus]

GenBank: AID69223

Allele #	Start	End	Peptide Length	Peptide
H-2-Kb 1	601	612	12	VTYGKNQVIMLL
H-2-Kb 1	681	691	11	ILYYYELYPTM
H-2-Kd 1	13	25	13	RYQPRPWTPRSTI
H-2-Kd 1	195	203	9	QYSGGRFTI
H-2-Kd 1	308	316	9	YYQLLQASL
H-2-Ld 1	894	904	11	YPFMWGGAYCF
H-2-Db 1	1140	1148	9	HSMTNAVTI
H-2-Db 1	45	53	9	ISAVNKLTM
H-2-Kb 1	680	687	8	IILYYYEL
H-2-Kb 1	808	815	8	SAYEHVTV
H-2-Kb 1	601	612	12	VTYGKNQVIMLL
H-2-Ld 1	772	780	9	IPLAALIVL
H-2-Ld 1	728	736	9	TPGATVPFL
H-2-Ld 1	728	737	10	TPGATVPFLL
H-2-Ld 1	866	877	12	SPYVKCCGTAEC
H-2-Ld 1	414	425	12	APCTITGTMGHF
H-2-Kd 1	1198	1206	9	NYPASHTTL
H-2-Ld 1	763	774	12	QPLFWLQALIPL

Predicted peptide list of DENV derived peptides:

These peptides were synthesized

B-cell epitopes for polyprotein [Dengue virus 2]

>gi|356960980|gb|AET43250.1| polyprotein [Dengue virus 2]

	Start	End	Sequence	length
	1481	1495	DVPSPPPVGKAELED	15
	2215	2226	EPEKQRTPQDNQ	12
	2593	2608	GLTKGGPGHEEPIPMS	16
	2850	2862	VDTRTQEPKEGTK	13
	2639	2649	GESSPNPTIEA	11

MHC-I Binding Prediction Results: (Ordered Peptides)
Polyprotein [Dengue virus 2]
>gi|356960980|gb|AET43250.1| polyprotein [Dengue virus 2]

Haplotype	no.	Start	End	lth.	Sequence
H-2-Db	1	2309	2317	9	TAIANQATV
H-2-Db	1	2728	2736	9	RMLINRFTM
H-2-Db	1	284	292	9	IGISNRDFV
H-2-Db	1	2342	2350	9	YSQVNPITL
H-2-Db	1	3301	3309	9	LAVWNRVWI
H-2-Kd	1	1269	1277	9	KYQLAVTIM
H-2-Kd	1	1635	1647	13	AYVSAIAQTEKSI
H-2-Kd	1	1946	1954	9	QYIYMGEPL
H-2-Kd	1	2471	2479	9	SYLAGAGLL
H-2-Kd	1	3248	3256	9	SYAQMWTLM
H-2-Kb	1	51	59	9	VAFLRFLTI
H-2-Kb	1	163	170	8	ITYKCPLL
H-2-Kb	1	1163	1170	8	VAVSFVTL
H-2-Kb	1	698	705	8	TAWDFGSL
H-2-Kb	1	2185	2193	9	SILLWYAQI

MHC-I Binding Prediction Results
>gi|376341628|gb|AFB35200.1| alpha-gliadin [*Triticum aestivum*]

Some peptides that can be used for testing in a mouse model were also predicted. However, further studies were not performed on these peptides

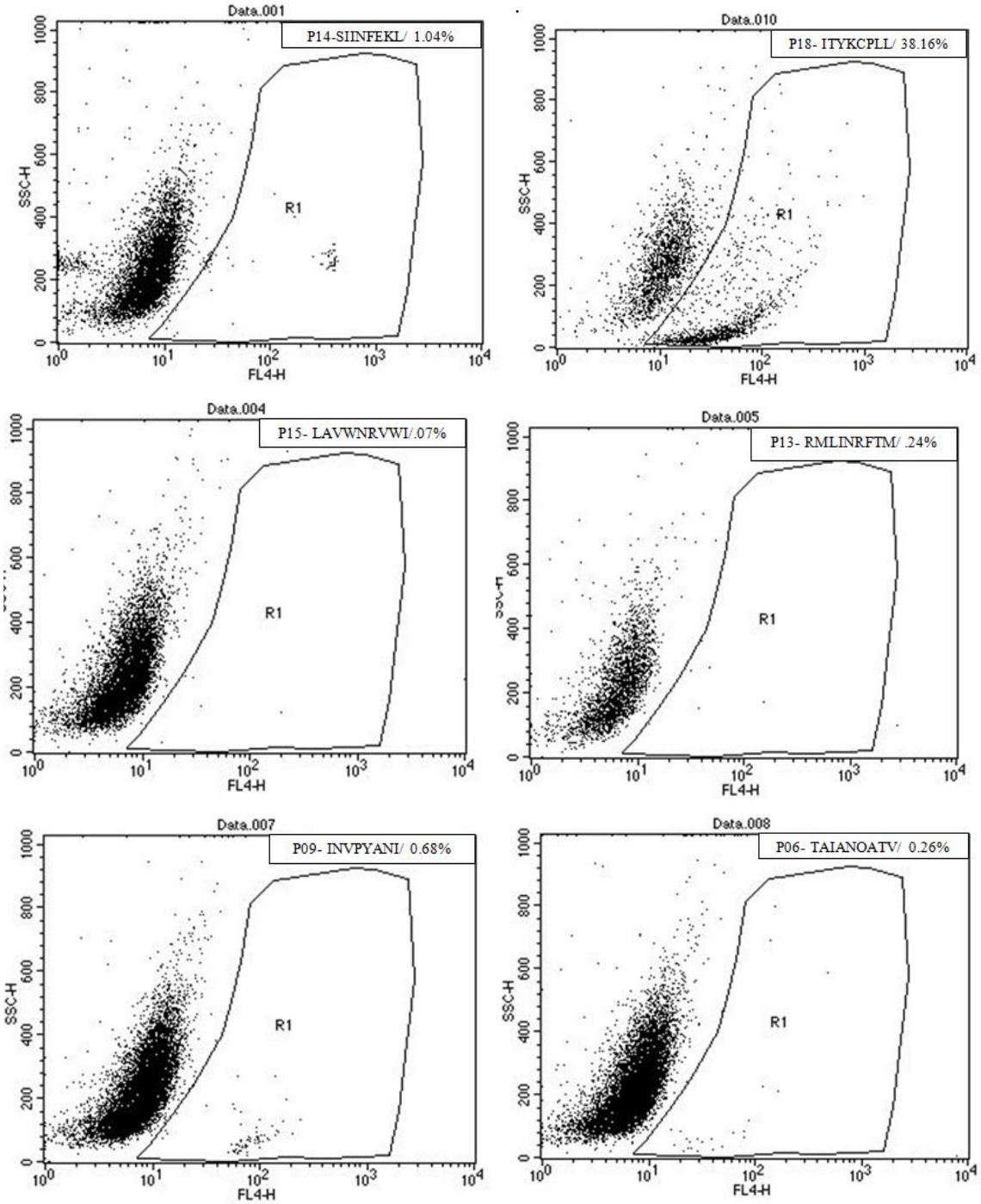
H-2-Kb	1	288	295	8	INVPYANI
H-2-Kd	1	8	16	9	TILAMATTI
H-2-Db	1	182	191	10	VSLVSSLVSI

All the predictions were also done for Chikungunya virus but peptides were not synthesized for testing, List of peptide is as follows. (Arrange the list where it belongs)

MHC stabilization on RMA/s cells by predicted peptides:

RMA/s cells lack in TAP molecules and therefore express very low levels on their surface. However, if a peptide is added during their culturing, the peptide could get access to newly synthesized MHC molecule via a retrograde transport and eventually leading to stabilization of the MHC molecule on the surface. This property of RMA cells was used to ascertain whether our predicted peptides were able to stabilize MHC I on their surface. Following FACS plots show the ability of different peptides to stabilize MHC on their surface as testing by surface staining of these cells with APC anti-MHC class I antibody.

2.3.1 RMA/s cell line peptide stabilization assay:



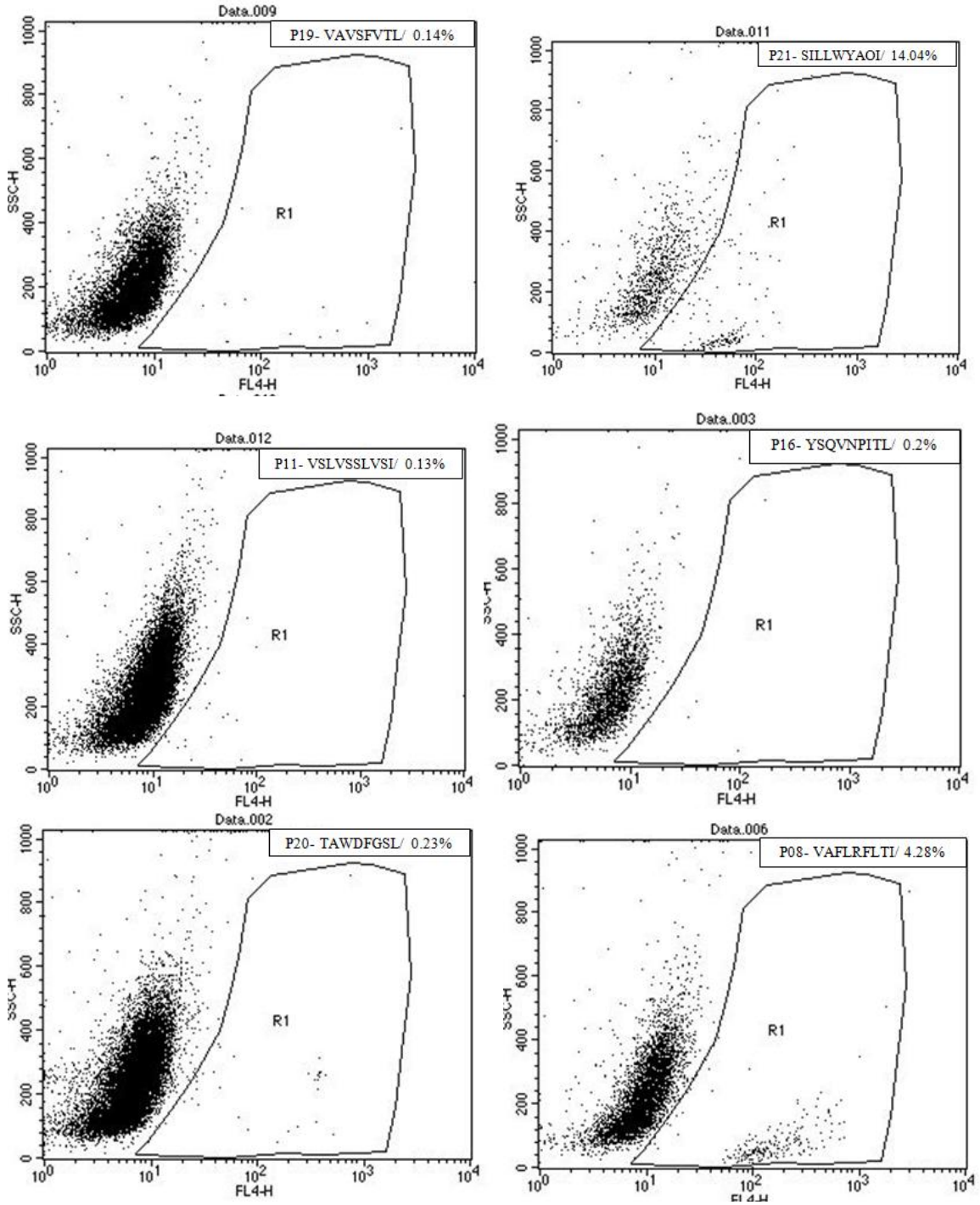


Fig2.2 RMA/S Cell population shift in FL4 channel with their respective peptide as mention in each image at concentration 800microgm/ml for 5 hrs incubation. {stained with anti-mouse H2Kb-APC}.

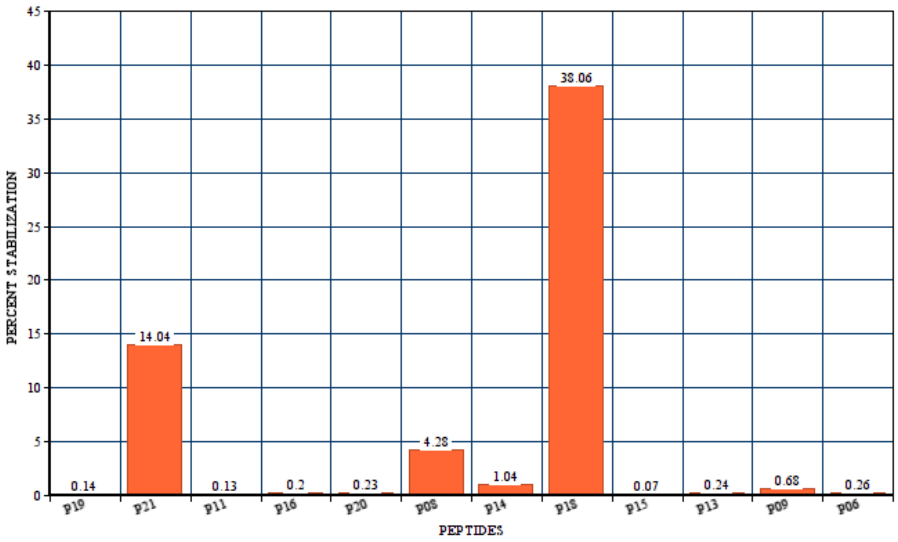


Fig. 2.3 Relative peptide stabilization on MHC-I molecules(H2Kb)

As shown by FACS plots (Fig 2.2) and the bar graphs (Fig 2.3) dengue virus derived peptides P18 (ITYKCPLL) P21 (SILLWYAQI), P08 (VAFLRFLTI) were able to stabilize MHC I on the surface of RMA/s cells in a decreasing order. To a lesser degree, peptide 14 ( ) and peptide 9 (INVPYANI) were also able to stabilize MHC.

ELISA using peptide exchange approach to determine the ability of peptides to bind MHC class:

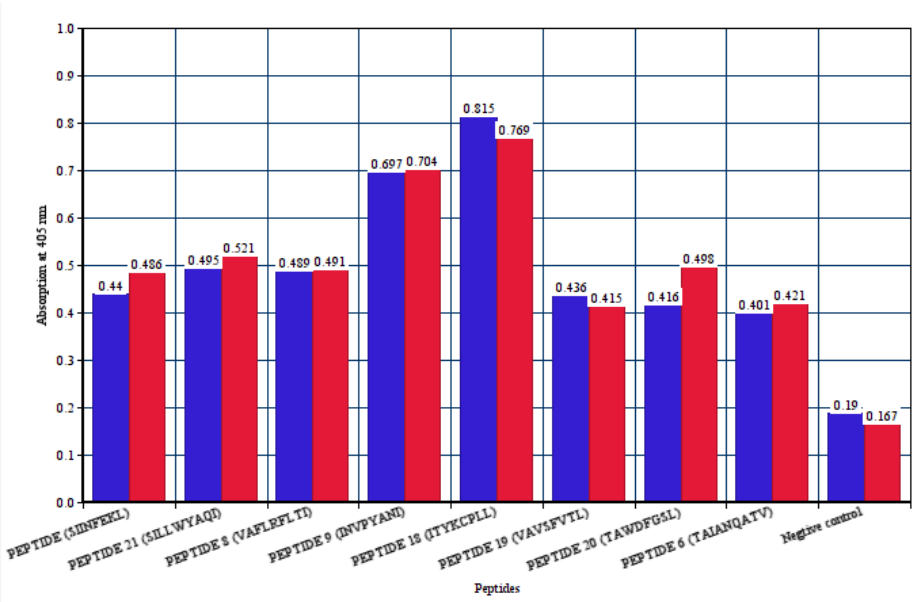


Fig. 2.4. First peptide (SIINFEKL) acting as a control for other H2Kb specific peptides showing absorption and the stabilization of MHC-I molecule for their respective peptides as denoted at X axis.

In second set of experiments, an ELISA based approach was used to validate the predicted peptides to establish whether or not they are able to bind MHC molecule. For this an MHC monomer were incubated with a conditional ligand approach was used as described in materials and method section.

As shown in figure 2.5, peptides18 (**ITYKCPLL**), peptide 9 (**INVPYANI**), peptide 21(**SILLWYAQI**) fared better than SIINFEKL with equimolar concentrations confirming the results from RMA/s cells and further suggesting that indeed out of those peptides tested, at least three peptides are immunogenic and could be considered for in vivo studies.

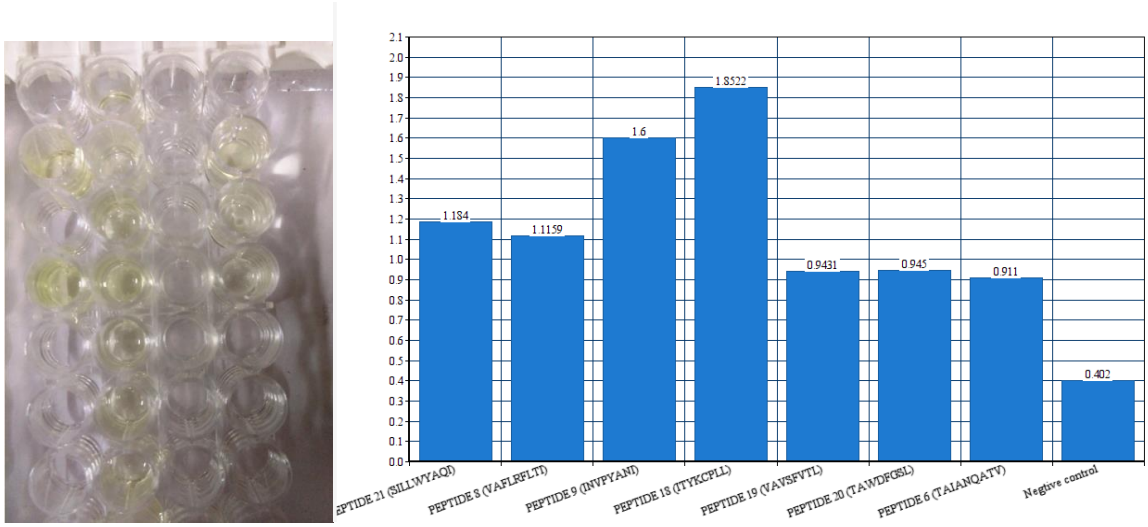


Fig. 2.5 Left panel showns a picture of ELISA strips demonstrating how an ELISA based approach works for determining the MHC binding ability of a peptide upon UV mediated conditional ligand displacement. Right panel showns a bar graph to depict a fold change in OD value for respective peptides as compared to a known good binder for MHC class I molecule.

2.4 Conclusion

MHC stabilization assay using RMA/S cell line and ELISA based assays were used to validate potentially good binders as predicted by bioinformatics tools. Some of the peptides were validated to bind to MHC in both assays while other peptides showed discordant results. It could be either due to the sensitivity of the assay or the quality of

synthesized peptides. Thus, for ELISA very small quantities of peptides were used while for cellular stabilization of MHC a larger concentration of peptides were required and if there are some contaminant inhibitors were present in the peptide preparation, they could skew the results as we observed in both the assays. None the less this study predicted some novel peptides of DENV proteins that could potentially be used for in vivo studies to establish their value towards a subunit vaccines and deciphering immunological events in the immunity and immunopathology caused by DENV. Peptides for CHIKV were also predicted but their validity was not established and could be part of future studies.

Some of predicted peptides stabilized the MHC-I monomer and gave positive results. Now these monomers can be exploited for making MHC tetramer so that Specific T- cells against respective peptides can be detected in a system.

Different online tools and software follows different approaches to generate different lists of immunogenic peptides such as SYFPEITHI uses the concept of anchor residue and preferred residue while IEDB ANN uses artificial neural network and SMMPMBEC uses position specific scoring matrices. There is no guarantee that a predicted epitopes will also be a good epitope in experimental conditions. High throughput studies are always better to conduct but in limited resource condition we can use an approach to increase the probability to find some epitopes but using this approach, we validated H2Kb and H2Db peptides in-vitro condition.

Conservation analysis was necessary as if we select an epitope which shows sequence similarity with host then that peptide will not work in spite of the fact that how good is that epitope. The peptide will be identified as a self and no immunological response will be generated. Sequence should also not similar to other organism, as epitope will not be specific.

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