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Design and testing of a highly conserved human rotavirus VP8* immunogenic peptide with potential for vaccine development

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ABSTRACT

Rotavirus infection of young children particularly below five years of age resulting in severe diarhoea, is the cause of a large number of infant deaths all over the world, more so in developing countries like India. Vaccines developed against this infection in the last two decades have shown mixed results with some of them leading to complications. Oral vaccines have not been very effective in India. Significant diversity has been found in circulating virus strains in India. Development of a vaccine against diverse genetic variants of the different strains would go a long way in reducing the incidence of infection in developing countries.

Success of such a vaccine would depend to a large extent on the antigenic peptide to be used in antibody production. The non-glycosylated protein VP4 on the surface capsid of the virus is important in rota viral immunogenicity and the major antigenic site(s) responsible for neutralization of the virus via VP4 is in the VP8* subunit of VP4. It is necessary that the peptide should be very specific and a peptide sequence which would stimulate both the T and B immunogenic cells would provide maximum protection against the virus. Advanced computational techniques and existing databases of sequences of the VP4 protein of rotavirus help in identification of such specific sequences. Using an *in silico* approach we have identified a highly conserved VP8* subunit of the VP4 surface protein of rotavirus which shows both T and B cell processivity and is also non-allergenic. This sub-unit could be used in *in vivo* models for induction of antibodies.

1. Introduction

Rota virus infection is a common cause for childhood diarrhoea worldwide. Mucosal immunity is important in prevention of the rota gastroenteritis (Settembre et al., 2011; Offit, 1996; Matson, 1996; Yuan and Saif, 2002; Franco et al., 2006; Desselberger and Huppertz, 2011) and oral vaccines are considered effective because they may elicit both mucosal and systemic immune responses.

The rotavirus genome consists of eleven segments of dsRNA which encode for twelve proteins. The NSP1-NSP6 proteins are nonstructural and appear only after infecting the host cells. VP1-VP4, VP6 and VP7 are structural proteins. VP4, VP7, VP6, NSP2 and NSP4 have been found to be immunogenic, the latter two less than the former three (Ishida et al., 1996). The non-glycosylated protein VP4 is important in rota viral immunogenicity (Hoshino and Kapikian, 2000). The major antigenic site(s) responsible for neutralization of the virus via VP4, is in the VP8* subunit of VP4 (Larralde et al., 1991). Antibodies present in the serum of rota infected children react against the variable VP8* region corresponding to different P[8] human rotavirus lineages (Contreras et al., 2011). It is only when the VP4 is cleaved by intestinal proteases into VP8* and VP5*, that the virus becomes infective (Rodrı´guez et al., 2014). It is the lectin like globular domain of the VP8*, residues 65–224 which interact with the host receptor and is involved in the attachment of the virus to the host cell (Rodrı´guez et al., 2014).

Several rotavirus candidate vaccines have been found to be useful in reduction of rota gastroenteritis (O'Ryan et al., 2015). Among the live attenuated oral vaccines, only two are licensed (Ward et al., 2008). Rotarix is a monovalent vaccine containing the attenuated human G1P [8] rotavirus strain 89–12 (Kulkarni et al., 2014). Rotateq is a pentavalent vaccine containing five reassortants representing the common human VP7 types, G1-4 and the most commonVP4 type, P[8] (Kang, 2016). Rotavac composed of 116E, a natural reassortant, deriving its VP4 gene segment from a bovine rotavirus and the other 10 genes from a human strain was introduced in India in 2016 (Bhandari et al., 2014).

Though, Rotateq and Rotarix have had a good impact in reducing

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rotavirus infection in most parts of the world, their clinical effectiveness has been found consistently to be lower in developing countries (Patel et al., 2012; Lamberti et al., 2016; Parashar et al., 2016). Furthermore, reports of adverse effects of intussusception have led to search for alternatives.

Exploration of alternative approaches led to the discovery that parenteral administration of a subunit vaccine would induce a systemic immune response (Wen et al., 2012).

During the last decade several subunit vaccines against specific viral proteins have been proposed. Peptide vaccines focus on specific conserved epitope regions of pathogen proteins to induce immune response as the epitope alone is considered sufficient for development of an immune response (Bijker et al., 2007; Lin et al., 2013). Such peptides have the advantage that they exclude unnecessary proteins, which do not contribute to the protective immune response, yet would be responsible for allergenic/and or reactogenic responses (Li et al., 2014). Peptide vaccines have other advantages as well. They are economically affordable, require less time for development and can be administered in multivalent dosages. Subunit vaccines for several diseases (Langeveld et al., 1994; Wang et al., 1995; Monso et al., 2011; Nandy and Basak, 2016), are in different phases of clinical trials (Li et al., 2014; Thompson and Staats, 2011; Petrovsky and Aguilar, 2004).

In this study, we have employed a series of immunoinformatics tools for the identification of a specific conserved peptide sequence containing both B and T cell epitope from the VP8* subunit of VP4 surface protein of rotavirus A. An *in silico* check for the antigenicity of the region, predicted that the epitope could generate an immune response. Further, investigation of the peptide for immunogenicity in mice portrayed that the conserved epitope is immunogenic in nature. Overall, the present study helped in identification of novel conserved immunogenic epitope through application of immunoinformatics tools which may pave the way for development of novel peptide based vaccines for rotavirus A in near future.

2. Materials and methods

2.1. Databases and software tools used

Sequence variants of the VP4 protein of human rotavirus A were retrieved from NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) and UniProtKB database (http://www.uniprot.org). Multalin (http://multalin.toulouse.inra.fr/multalin/) was used to align the VP4 protein sequences and detect the conserved region. Antigenicity of the continuous conserved regions was determined by VaxiJen 2.0 (Doytchinova and Flower, 2007). The antigenic peptides selected were submitted to IEDB T cell epitope MHC2 tool (http://tools.iedb.org/mhcii/) and Propred (Singh and Raghava, 2001) for T cell epitope prediction. The antigenic peptides were also submitted to IEDB B cell epitope prediction tool Vita et al., 2010 to determine the best epitope. The peptide sequences which were common as both B cell and T cell epitopes were chosen to be assessed for allergenicity by web based AllerHunter server (Muh et al., 2009) and AlgPred (http://crdd.osdd.net/raghava/algpred/). The peptide was matched using PIR Peptide Match (https://research.bioinformatics.udel.edu/peptidematch/). Molecular docking for determination of HLA and epitope binding prediction was done by CABS-dock (http://biocomp.chem.uw.edu.pl/CABSdock) and RossettaFlexPepDock web server (Liu et al., 2014). Fig. 1 summarises the different steps used for the complete epitope peptide prediction.

2.2. Retrieval of VP4 protein

Sequence variants of the VP4 protein of human rotavirus A were retrieved from NCBI protein and UniProtKB database. A total of 390 fasta formatted sequences of the human rotavirus A VP4 protein were retrieved from GenBank of NCBI and UniProtKB. We have applied multiple sequence alignment MultAlin to remove duplicates and partial sequences from the dataset.

2.3. Prediction of the conserved region in VP4 protein

MultAlin was used to align the set of non-redundant VP4 protein sequences to detect the conserved regions.

2.4. Antigenicity

Each of the continuous regions/segments within the conserved region was subjected for assessment of antigenicity by VaxiJen 2.0, a server for alignment-independent prediction of protective antigens and which allows antigen classification solely based on the physicochemical properties of proteins (Doytchinova and Flower, 2007).

2.5. B cell epitope prediction

B cell epitope prediction tool (Vita et al., 2010) from Immune Epitope Database (IEDB) was used to determine the B cell antigenicity and determine the best epitope. Kolasker and Tongaonkar antigenicity scale (Kolaskar and Tongaonkar, 1990) and Emini surface accessibility prediction tool (Emini et al., 1985) were also used. Kolaskar and Tongaonkar antigenicity method was applied using a threshold of 1.000. All values higher than one are antigenic determinants. It is known that flexibility is directly proportional to antigenicity of the peptide. Karplus and Schultz flexibility prediction tool (Karplus and Schulz, 1985) was used to predict the flexibility of the conserved peptide.

Bepipred linear epitope prediction analysis (Larsen et al., 2006) which predicts the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method and the Chou and Fasman beta-turn prediction tool (Chou and Fasman, 1978) were used to determine the β turn regions as it is known that antigenic regions belong to β turn regions and these regions are also highly hydrophilic (one of the required characteristics). The hydrophilicity was predicted by Parkers hydrophilicity tool (Parker et al., 1986).

2.6. T cell epitope prediction

The antigenic peptides selected were submitted to IEDB T cell epitope MHC2 tool (http://tools.iedb.org/mhcii/) and Propred (Singh and Raghava, 2001) for T cell epitope prediction. To identify an antibody production inducing peptide, the most widely used tools *i.e.*, IEDB (http://tools.iedb.org/mhcii/) and Propred `were employed for the determination of MHC II binding predictions. The threshold was set at 4. All alleles of MHC II were chosen for peptide binding prediction. In case of IEDB, for the peptide binding predictions, a reference set of alleles were chosen. The lowest consensus scores of the peptides were chosen to be the best binders as according to IEDB server, a lower percentile rank indicates higher affinity. Two different tools were used to obtain reliable results. The common region procured by both the tools was selected as T cell epitope.

2.7. Alleregenicity assessment

The peptide sequences which were common as both B cell and T cell epitopes were chosen for assessment of allergenicity, through the web based AllerHunter server (Muh et al., 2009). Apart from AllerHunter, we have also employed AlgPred tool to check allergenicity of the conserved peptides. AlgPred allows the prediction of allergens based on similarity of known epitope with any region of protein. The peptide was matched using PIR Peptide Match to show that the sequence is present only in VP4 region.

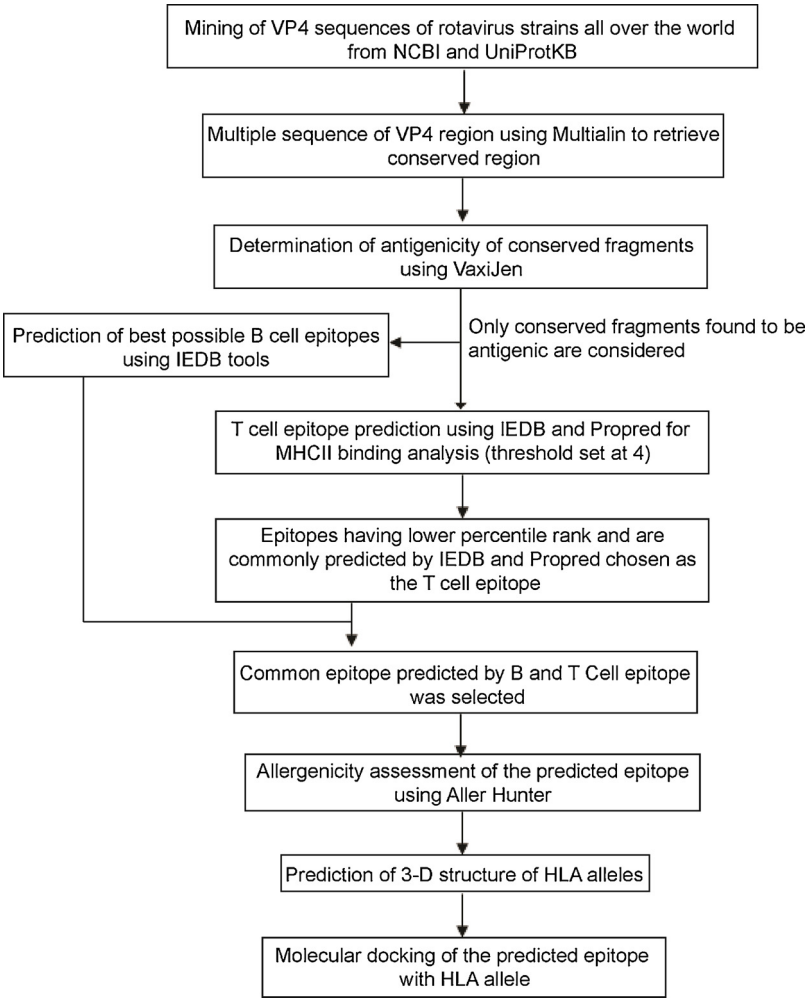

Fig. 1. Protocol summarizing immunoinformatics approach for prediction of promiscuous epitope from VP4 sequences of Rotavirus A.

2.8. Determination of the 3D structures of the predicted epitope and human leucocyte antigen (HLA) alleles

The predicted epitope was used for molecular docking with the respective best fitted HLA alleles. 3D structures of the peptide and HLA alleles were also predicted (in some cases crystal structure of HLA alleles were used). The crystal structure of HLA-allele *i.e.,* C7DQA03:01/dab 03:02 (PDB ID: 4D8P) (Tollefsen et al., 2012) and DRB3.02:02 (PDB ID: 3C5J) (Wisedchaisri et al., 2008) was downloaded from RCSB PDB (www.rcsb.org), which was used for docking with the predicted epitope peptide. In the absence of a 3-D structure of the HLA allele, a theoretical approach was used to predict the 3-D structure. The protein sequences of HLA alleles (DRB1:04:11-04:21, DRB1:04:22-04:33 and DRB1:04:34-04:45) were downloaded from UniProtKB. As all the above HLA alleles belong to DRB1, only one representative allele *i.e.*, DRB1:04:11-04:21 was chosen for 3-Dimensional model building. Suitable templates were identified using BLAST against PDB (as listed in Supplementary File 1. Table S1). Using optimized target-template alignment, 20 models for each HLA allele (as mentioned above) were generated using Modeller (Eswar et al., 2006) where model with the lowest DOPE score was selected for loop and side chain refinement. The optimized model was submitted to various model validation servers to gauge different stereochemical parameters. Ramachandran plot embedded in PROCHECK tool (Laskowski et al., 1993) was used to evaluate the phi/psi distribution of each amino acid in the model and to quantify amino acids into favourable and non-favourable zones. ERRAT (http://services.mbi.ucla.edu/ERRAT/) and Verfify-3D (Luthy et al., 1992) were employed to estimate statistics of non-bonded interactions between different atom types and compatibility of an atomic model (3D) with its own amino acid sequence (1D). The PROSA web tool, which uses a Z-score (Wiederstein and Sippl, 2007) was used to evaluate the overall model quality of the modelled structures. Finally, the root mean square deviation (RMSD) between the Cα atoms of the model and respective templates was calculated by structural superimpositions using PyMOL (https://www.pymol.org).

2.9. Molecular docking for HLA-epitope binding prediction

The CABS-dock (http://biocomp.chem.uw.edu.pl/CABSdock) and Rosetta FlexPepDock (http://flexpepdock.furmanlab.cs.huji.ac.il/) servers were used for molecular docking for prediction of HLA and epitope binding of the chosen epitope peptide with crystal and/or modelled HLA allele structures. Best ranked poses screened from CABS-dock were subsequently refined through FlexPepDock. The protein-peptide interactions (of the best docked poses in each case) were visualized using BIOVIA Discovery Studio VisualizerV4.5.

2.10. Synthesis of peptide

The identified peptide sequence was synthesized by Sigma Aldrich.

2.11. Animal immunization with synthesized peptide

Ethical clearance for animal experiments was obtained from the

Institute Animal Ethics Committee. Two groups of 6 to 8 week old naïve female Balb/c mice 9 (six and five in each group respectively) were obtained from the in house animal facility and maintained and cared for as per the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment & Forests (Animal Welfare Division), Government of India.

The immunogen peptide, containing 80 µg of protein was mixed in Freund's complete adjuvant and Freund's incomplete adjuvant and was injected subcutaneously to one group (Test group-6) at 0 and 14 days respectively. Booster dose of the peptide only in phosphate-buffered saline (PBS), was given on day 28 after the initial immunization. PBS instead of the peptide was injected subcutaneously at the same time periods, into the other group of mice (Negative control group-5). Blood samples were collected from each mouse before the first immunization and on 14, 28, 35 and 42 days after the first immunization. Serum was separated and stored at −80 °C till further testing.

2.12. Quantitative detection of peptide-specific antibody response

Antibody levels in serum were assessed by Enzyme linked immunosorbent assay (ELISA). Each well of the ELISA plate was coated with 2.5 µg of the peptide in 50 µl of carbonate bicarbonate buffer (pH 9.6). The ELISA plate was incubated overnight at 40 °C. After washing with phosphate buffered saline (PBS) three times, the wells were blocked with 1% skimmed milk in PBS at 37 °C for 1 h. After 3 washes, 20 fold serially diluted respective serum samples were added and incubated at 37 °C for one and a half hours. After 3 times washing, Horse Radish Peroxidase (HRP) conjugated goat anti mouse IgG was added and incubated at 37 °C for one and a half hour. The enzyme activity was measured using ortho phenylene diamine (P1526, Sigma). Absorbance was read at 492 nm using an automated ELISA reader.

3. Results

3.1. Protein sequence retrieval

After removal of duplicates as well as short/fragment sequences, a total of 363 sequences of VP4 were finally subjected to multiple sequence alignment in MultAlign. We included the vaccine sequences of Rotarix (GenBank Accession No: JN849113) and RotaTeq (GenBank Accession No: GU565044), the two licensed rota virus vaccines, as well.

3.2. Conservancy of VP8* region in the VP4 protein

From the output of multiple sequence alignment, Leu65-Cys226 region was found to be conserved. From the conserved region, seven continuous conserved sequences were identified (as shown in Supplementary File 2. Fig. S1). The continuous conserved sequences span from Leu65-Phe74, Ser86-94Glu, Thr96-Val112, Phe122-Val130, Asn154-Asp161, Lys168-Pro189, and Ile202-Cys226.

3.3. Antigenic protein determination

Out of the seven conserved regions, the span Phe122-Val130 and Ile202-Cys226 were found to be antigenic with a score of 0.6401 and 0.4466 respectively (Table 1). In the amino acid sequence Phe122-Val130, position Asn125 was included instead of Ser125 as it was the most conserved residue found in majority of the VP8* sequences submitted till date, unlike Ser125 which was present only in a few of the isolates. These two antigenic peptide sequences were used for further analysis.

3.4. B cell epitope prediction

One epitope 205–211 amino acid region was found to be antigenic along with regions 214–215 amino acids and 217–219 amino acids, as summarized in Fig. 2. The region consisting of 211–216 amino acid residues (RSQESKC) was predicted to be more accessible. The region consisting of 207–215 amino acid residues was predicted to be most flexible. The region consisting of 212–223 amino acid residues was predicted to be the B turn regions. Position 211–222 amino acid residues were hydrophilic. By using BepiPred-2.0, the linear epitope prediction tool, the region consisting of 212–218 amino acid residues was predicted as the B cell epitope. The default threshold was 0.35.

Several epitopes were predicted by these algorithms, but only the epitope sequence region that was found with at least 90% overlap between all the IEDB B-cell epitope prediction tools and Bepipred prediction tool was chosen as the desired B cell epitope (Table 2A–F). By analyzing all the results, we predicted that the 205–218 (EFYIIPRSQESKCN) amino acid region was good for eliciting the required B cell response.

3.5. T cell epitope prediction – MHC-II binding prediction

Both the conserved regions were submitted to the IEDB epitope prediction server for T-cell MHC-II binding predictions (Table 3). The peptide Phe122-Val130 did not show any binding, while the peptide Ile202-Cys226 showed binding to the reference set of alleles (covers most of the population) as suggested by IEDB server. The binding scores of each of the peptides (IEDB) are illustrated in Table 3. Results of ProPred analysis of the peptide for binding to HLA–DRB alleles are given in supplementary File 3 (Table S2). The 206–216 (FYIIPRSQESK) amino acid region (SEQ1) was found to be common among the two different tools (*i.e.*, IEDB and Propred) used for MHC II binding and also bound to the maximum number of alleles. Propred revealed binding to 22 alleles while IEDB revealed binding to 27 alleles (Table 3). The percentile rank in IEDB tool revealed very lower values in comparison to the other regions in the peptide which suggested a stronger binding.

3.6. Allergenicity assessment

The sequence-based allergenicity prediction was precisely

Table 1
Out of the seven conserved regions, the span Phe122-Val130 and Ile202-Cys226 were found to be antigenic with a score of 0.6401 and 0.4466 respectively.

Sl. No.	Conserved Region (Position)	Vaxigen score (Prediction Results)
1	LDGPYQPTTF (65–74)	0.2573 (Probable non antigen)
2	SNTNGVVYE (86-94)	−0.2416 (Probable non antigen)
3	TNNSDFWTAVVAVEPHV (96–112)	0.3799 (Probable non antigen)
4	FGENKQFNV (122–130)	0.6401 (Probable antigen)
5	NRRTLTSD (154-161)	0.0606 (Probable non antigen)
6	LKYGGRVWTFHGETPRATTDSS (168–189)	−0.2451 (Probable non antigen)
7	IHSEFYIIPRSQESKCNEYINNGLP (202–226)	0.4466 (Probable antigen)

Antigenicity assessment of the conserved regions of VP8 region of Rota Virus strains.

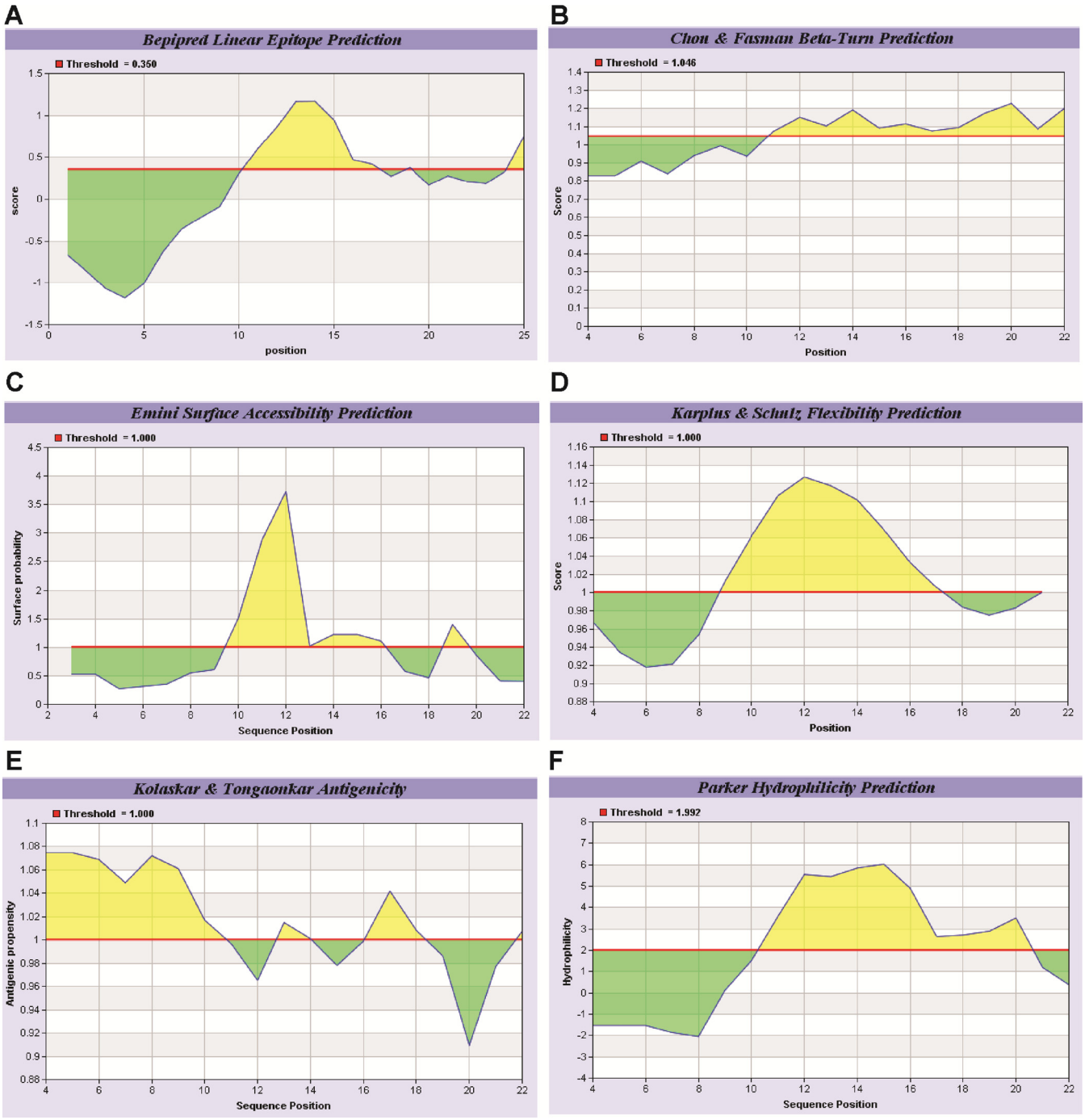

Fig. 2. B cell epitope prediction using Immune Epitope Database IEDB, B-cell epitope prediction tools and Bepipred on the conserved protein sequence. The x-axis and y-axis represent the position and score, respectively. The red horizontal line indicates cut-off. The yellow region indicates region of peptide above threshold value while the green region indicates region of peptide below threshold values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

calculated using the AllerHunter tool, and the predicted queried epitope allergenicity score was 0 (sensitivity = 93.0%, specificity = 79.4%). Hence, the peptide epitope was predicted to be a non-allergen. The findings of AllerHunter were well supported with the results of AlgPred which signifies non-allergenic nature of the epitope. The PIR Peptide Match also showed the sequence to be only present in VP4.

3.7. Molecular docking analysis

3.7.1. 3-Dimensional modeling of HLA alleles

The 3-dimensional structure of drb1:04:11–04:21 was generated using Modeller, based on the available templates (*i.e.*, 1AQD, 3PDO and 2WBJ) having sequence identity of 87%, 87% and 85% respectively (as shown in Supplementary File 1. Table S1). The model with lowest DOPE score was selected and was subjected to a model validation process through PROCHECK, Verify-3D, ERRAT and ProSA for their stereo-chemical quality and reliability. Ramachandran plot statistics revealed that 99.0% of residues are found in the favorable region (Fig. 3) and ensured that modeled DRB1 is of good quality. ERRAT score report revealed that overall model quality of the predicted structure was 73.718, within the acceptable range. ProSA-web Z-score (Z score of −3.91) reports revealed that the modeled DRB1 HLA allele structure

Table 2
Several epitopes were predicted by these algorithms, but only the epitope sequence region that was found with at least 90% overlap between all the IEDB B-cell epitope prediction tools and Bepipred prediction tool was chosen as the desired B cell epitope.

(A) Chou & Fasman Beta-Turn Prediction

SI.no	Peptide start position	Peptide end position	Peptide Score	Score
1	1	7	IHS**E**FYI	0.829 (minimum)
2	2	8	HSE**F**YII	0.829
3	3	9	SEF**Y**IIP	0.91
4	4	10	EFY**I**IPR	0.841
5	5	11	FYI**I**PRS	0.94
6	6	12	YII**P**RSQ	0.994
7	7	13	IIP**R**SQE	0.937
8	8	14	IPR**S**QES	1.074
9	9	15	PRS**Q**ESK	1.151
10	10	16	RSQ**E**SKC	1.104
11	11	17	SQE**S**KCN	1.191
12	12	18	QES**K**CNE	1.093
13	13	19	ESK**C**NEY	1.116
14	14	20	SKC**N**EYI	1.077
15	15	21	KCN**E**YIN	1.096
16	16	22	CNE**Y**INN	1.174
17	17	23	NEY**I**NNG	1.227 (maximum)
18	18	24	EYI**N**NGL	1.089
19	19	25	YIN**N**GLP	1.2

(B) Emini Surface Accessibility Prediction results

SI.NO	Peptide start position	Peptide end position	Peptide	Score
1	1	6	IH**S**EFY	0.531
2	2	7	HS**E**FYI	0.531
3	3	8	SE**F**YII	0.274 (minimum)
4	4	9	EFY**I**IP	0.316
5	5	10	FYI**I**PR	0.357
6	6	11	YII**P**RS	0.553
7	7	12	II**P**RSQ	0.611
8	8	13	IP**R**SQE	1.509
9	9	14	PR**S**QES	2.885
10	10	15	RS**Q**ESK	3.731 (maximum)
11	11	16	SQ**E**SKC	1.021
12	12	17	QE**S**KCN	1.225
13	13	18	ES**K**CNE	1.225
14	14	19	SK**C**NEY	1.109
15	15	20	KC**N**EYI	0.58
16	16	21	CN**E**YIN	0.466
17	17	22	NE**Y**INN	1.399
18	18	23	EY**I**NNG	0.861
19	19	24	YI**N**NGL	0.41
20	20	25	IN**N**GLP	0.405

(C) Karplus and Schultz flexibility prediction

SI.NO	Peptide start position	Peptide end position	Peptide	Score
1	1	7	IHS**E**FYI	0.967
2	2	8	HSE**F**YII	0.934
3	3	9	SEF**Y**IIP	0.918 (minimum)
4	4	10	EFY**I**IPR	0.921
5	5	11	FYI**I**PRS	0.954
6	6	12	YII**P**RSQ	1.013
7	7	13	II**P**RSQE	1.062
8	8	14	IPR**S**QES	1.106
9	9	15	PRS**Q**ESK	1.127 (maximum)
10	10	16	RSQ**E**SKC	1.117
11	11	17	SQE**S**KCN	1.102
12	12	18	QES**K**CNE	1.07

(*continued on next page*)

Table 2 (*continued*)

(C) Karplus and Schultz flexibility prediction

SI.NO	Peptide start position	Peptide end position	Peptide	Score
13	13	19	ESK**C**NEY	1.034
14	14	20	SKC**N**EYI	1.006
15	15	21	KCN**E**YIN	0.984
16	16	22	CNE**Y**INN	0.975
17	17	23	NEY**I**NNG	0.983
18	18	24	EYI**N**NGL	1

(D) Kolasker and Tongaonkar Antigenicity prediction

SI.NO	Peptide start position	Peptide end position	Peptide	Score
1	1	7	IHS**E**FYI	1.075 (maximum)
2	2	8	HSE**F**YII	1.075 (maximum)
3	3	9	SEF**Y**IIP	1.069
4	4	10	EFY**I**IPR	1.049
5	5	11	FYI**I**PRS	1.072
6	6	12	YII**P**RSQ	1.061
7	7	13	IIP**R**SQE	1.017
8	8	14	IPR**S**QES	0.997
9	9	15	PRS**Q**ESK	0.965
10	10	16	RSQ**E**SKC	1.015
11	11	17	SQE**S**KCN	1.001
12	12	18	QES**K**CNE	0.978
13	13	19	ESK**C**NEY	0.999
14	14	20	SKC**N**EYI	1.042
15	15	21	KCN**E**YIN	1.008
16	16	22	CNE**Y**INN	0.986
17	17	23	NEY**I**NNG	0.909 (minimum)
18	18	24	EYI**N**NGL	0.977
19	19	25	YIN**N**GLP	1.008

(E) Parker Hydrophilicity prediction results

Si.no	Peptide start position	Peptide end position	Peptide	Score
1	1	7	IHS**E**FYI	−1.529
2	2	8	HSE**F**YII	−1.529
3	3	9	SEF**Y**IIP	−1.529
4	4	10	EFY**I**IPR	−1.857
5	5	11	FYI**I**PRS	−2.043 (minimum)
6	6	12	YII**P**RSQ	0.129
7	7	13	IIP**R**SQE	1.514
8	8	14	IPR**S**QES	3.586
9	9	15	PRS**Q**ESK	5.543
10	10	16	RSQ**E**SKC	5.443
11	11	17	SQE**S**KCN	5.843
12	12	18	QES**K**CNE	6.029 (maximum)
13	13	19	ESK**C**NEY	4.9
14	14	20	SKC**N**EYI	2.643
15	15	21	KCN**E**YIN	2.714
16	16	22	CNE**Y**INN	2.9
17	17	23	NEY**I**NNG	3.514
18	18	24	EYI**N**NGL	1.2
19	19	25	YIN**N**GLP	0.386

(F) Bepipred linear epitope prediction results

Position	Residue	Score	Assignment
1	**I**	−0.667	.
2	**H**	−0.866	.
3	**S**	−1.068	.
4	**E**	−1.177	.

(*continued on next page*)

Table 2 (*continued*)

(F) Bepipred linear epitope prediction results

Position	Residue	Score	Assignment
5	**F**	−1.008	.
6	**Y**	−0.629	.
7	**I**	−0.357	.
8	**I**	−0.22	.
9	**P**	−0.092	.
10	**R**	0.304	.
11	**S**	0.601	E
12	**Q**	0.858	E
13	**E**	1.163	E
14	**S**	1.166	E
15	**K**	0.945	E
16	**C**	0.47	E
17	**N**	0.417	E
18	**E**	0.273	.
19	**Y**	0.376	E
20	**I**	0.168	.
21	**N**	0.276	.
22	**N**	0.21	.
23	**G**	0.186	.
24	**L**	0.328	.
25	**P**	0.753	E

B cell epitope prediction results.
Sequence: IHSEFYIIPR SQESKCNEYI NNGLP.

was within the acceptable range of NMR and X-ray data (Fig. 3). Finally, the structural superimposition of Cα atoms of the models with respective Cα atoms of the templates revealed minimal deviation (within 1 Å) confirming the accuracy of the approach we used to determine the 3-D structures of HLA alleles.

3.8. Molecular docking of the predicted epitope with HLA alleles

The structural determinations of peptide complexes remain a challenging task. It is often more challenging when substantial conformational changes occur during the binding process. Hence, to obtain a starting structure, we employed CABS-dock for predicting the peptide-HLA complexes as it allows competent modeling of full peptide flexibility and significant flexibility of a receptor. From the resultant clusters generated by CABS-dock, we screened the top ranked cluster *i.e.*, with highest cluster density, least RMSD and more of number elements in the cluster. The binding pose of the top ranked cluster for HLA, C7Dqa 03:01/dab 03:02, DRB: 3.02:02 and DRB1:04:11–04:21 with the

Table 3
Both the conserved regions were submitted to the IEDB epitope prediction server for T-cell MHC-II bindingpredictions.

Allele	Start	End	Peptide	Method used	Percentile rank
HLA-DRB1*11:01	5	19	FYIIPRSQESKCNEY	Consensus (smm/nn/sturniolo)	3.42
HLA-DRB1*08:02	5	19	FYIIPRSQESKCNEY	Consensus (smm/nn/sturniolo)	5.38
HLA-DRB5*01:01	5	19	FYIIPRSQESKCNEY	Consensus (smm/nn/sturniolo)	12.49
HLA-DRB1*03:01	5	19	FYIIPRSQESKCNEY	Consensus (smm/nn/sturniolo)	18.01
HLA-DRB1*12:01	5	19	FYIIPRSQESKCNEY	smm	18.32
HLA-DRB1*04:05	5	19	FYIIPRSQESKCNEY	Consensus (smm/nn/sturniolo)	24.71
HLA-DRB1*04:01	5	19	FYIIPRSQESKCNEY	Consensus (smm/nn/sturniolo)	26.88
HLA-DRB1*07:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	28.71
HLA-DRB4*01:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	36.22
HLA-DRB1*01:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	42.08
HLA-DPA1*02:01/DPB1*01:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	42.45
HLA-DRB1*09:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	42.86
HLA-DQA1*04:01/DQB1*04:02	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	49.28
HLA-DRB1*15:01	5	19	FYIIPRSQESKCNEY	Consensus (smm/nn/sturniolo)	49.4
HLA-DPA1*03:01/DPB1*04:02	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	50.38
HLA-DRB1*13:02	5	19	FYIIPRSQESKCNEY	Consensus (smm/nn/sturniolo)	51.72
HLA-DPA1*02:01/DPB1*14:01	5	19	FYIIPRSQESKCNEY	NetMHCIIpan	51.98
HLA-DQA1*05:01/DQB1*02:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	52.27
HLA-DPA1*01:03/DPB1*02:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	53.46
HLA-DQA1*03:01/DQB1*03:02	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	54.61
HLA-DRB3*02:02	5	19	FYIIPRSQESKCNEY	NetMHCIIpan	56.13
HLA-DQA1*05:01/DQB1*03:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	66.69
HLA-DPA1*01/DPB1*04:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	67.71
HLA-DPA1*02:01/DPB1*05:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	71.91
HLA-DRB3*01:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	75.22
HLA-DQA1*01:01/DQB1*05:01	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	84.86
HLA-DQA1*01:02/DQB1*06:02	5	19	FYIIPRSQESKCNEY	Consensus (comb.lib./smm/nn)	86.46

The binding scores of each of the peptides (IEDB) are illustrated in Table 3.
Propred revealed binding to 22 alleles while IEDB revealed binding to 27 alleles.

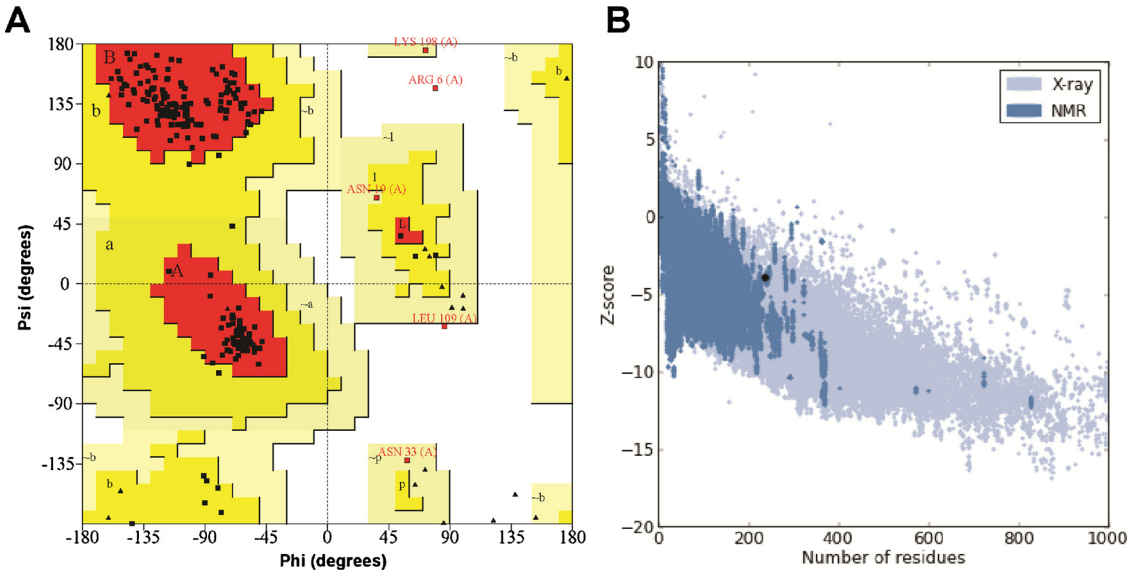

Fig. 3. Ramachandran plot statistics of the modelled drb1:04:11–04:21 shows that 99.0% of residues are found in the favorable region. ProSA-web Z-score (Z score of −3.91) indicates the quality of the predicted model of DRB1 HLA allele.

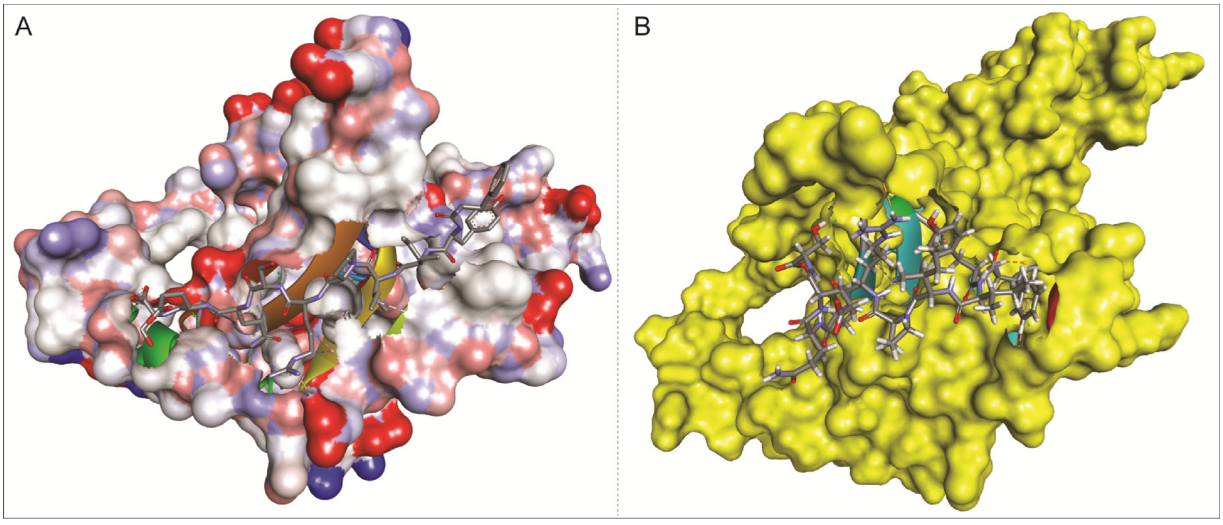

Fig. 4. Molecular docking of HLA Allele-Epitope. **(A)** HLA allele C7Dqa03:01/dab 03:02-peptide complex obtained from CABS-dock docking server. Epitope "FYIIPRSQES" bound to the binding groove with a total Rosetta score of −219.605 Kcal mol^{-1} **(B)** The predicted binding pattern of the epitope with HLA allele DRB 3.02:02 obtained from FlexPepDock docking server.

epitope has been displayed in Figs. 4–6. The top ranked clusters of CABS-dock server were subjected to refinement using FlexPepDock tool and ranked according to their Rosetta score (Supplementary File 4. Table S3). The predicted epitope SEQ1 was found to bind HLA, C7Dqa 03:01/dab 03:02, DRB: 3.02:02 and DRB1:04:11–04:21 with a total Rosetta score of −219.605, −327.886, and −369.124 kCal mol^{-1} respectively. The binding modes of epitope with three HLA alleles predicted using CABS-dock and FlexPepDock server have been illustrated in Figs. 4, 5 and 6.

3.9. Immunogenicity of the peptide SEQ1

Balb/c mice immunized with 3 doses of the peptide SEQ1 developed detectable neutralizing antibodies after the second dose of immunisation. Antibody levels in serum were found to be high during the period 28th to 42nd days. Antibody levels were higher in immunized animals than in control animals as shown in Fig. 7.

4. Discussion

Strategies for development of vaccines against rotavirus infection vary, from the use of live attenuated heterologous animal viruses and specific strains of the human virus to the use of viral proteins. There have also been attempts to identify a specific conserved portion of the viral protein and use the specific epitope as a vaccine. We have used an *in silico* approach to identify a highly conserved VP8* subunit of the VP4 surface protein of rotavirus and found that the conserved epitope SEQ1, has both T and B cell processivity, is antigenic and non-allergenic. The peptide was also found to be immunogenic in mice.

Recently, there have been attempts to use pieces of rotavirus proteins such as inactivated rotavirus particles, virus like particles and recombinant E. coli- expressed VP6 proteins as non-living vaccine candidates (O'Ryan et al., 2015). Full length or truncated VP8* proteins have been expressed and are found to elicit neutralizing antibodies in animals (Kovacs-Nolan and Mine, 2006; Xue et al., 2015). A clinical

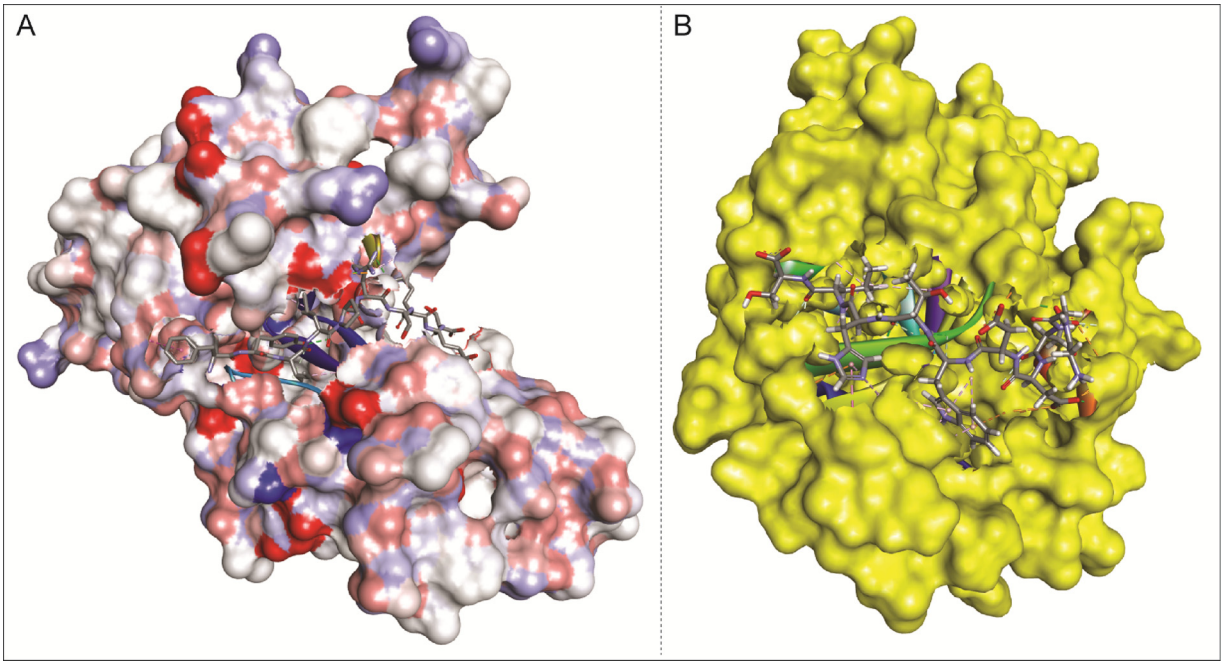

Fig. 5. Docking simulation results the predicted epitope with HLA allele structure. **(A)** The epitope bound to the binding groove of DRB: 3.02:02 with Rosetta score of -327.886 Kcal mol^{-1} obtained using CABS-dock and **(B)** The predicted binding mode of the epitope with HLA allele obtained using FlexPepDock server.

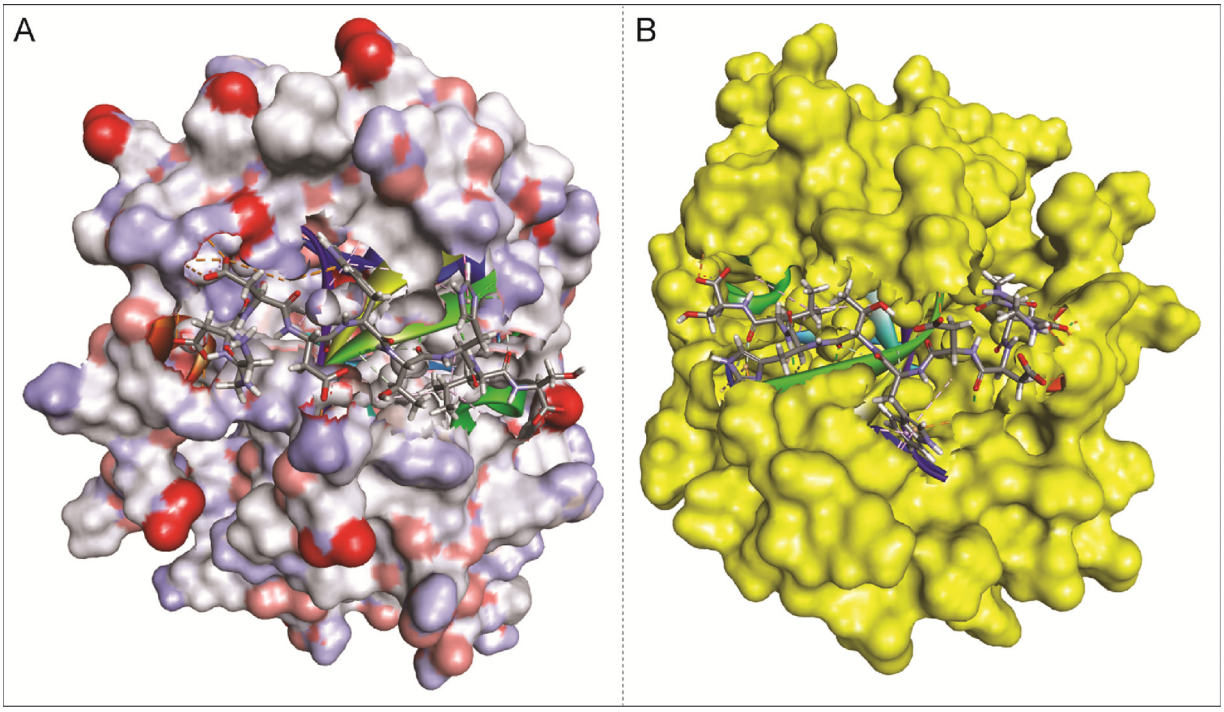

Fig. 6. (A) Molecular interaction of HLA-allele DRB1:04:11–04:21 with predicted epitope "FYIIPRSQES with Rosetta score of -369.124 mol^{-1} obtained using CABS-dock. **(B)** The binding mode of the promisocus epitope into the binding groove of HLA allele as predicted by FlexPepDock server.

trial with a P2-VP8* subunit vaccine revealed that the vaccine is well tolerated and evokes immune responses in humans as well (Fix et al., 2015). A disadvantage for this prospective vaccine candidate is that industrial preparation would be very expensive and time consuming.

Flexibility to match escape variants is easier with peptide vaccines comprised of the small synthetic protein fragments in comparison to classical protein vaccines like inactivated split virion or purified subunits and live attenuated vaccines. Production of synthetic peptide vaccines are controlled utilising pure production proccedures as opposed to the latter and overcomes the grave risk of contamination with extraneous agents and proteins of the production substrate with the latter (Huber et al., 2014).

There are large differences among the antigenic epitopes of the VP8* region of rota virus A strains circulating in different parts of the globe (Dormitzer et al., 2002). Most of the genetic variation is in the VP8* cleavage fragment of VP4 (Zeller et al., 2012). Amino acid variations in the antigenic epitopes of the VP8* region may affect efficacy of the vaccine (Kulkarni et al., 2014). Structural analysis has revealed that some amino acid differences are at the exposed regions of the antigenic epitopes of the VP8* region and could affect antigenicity

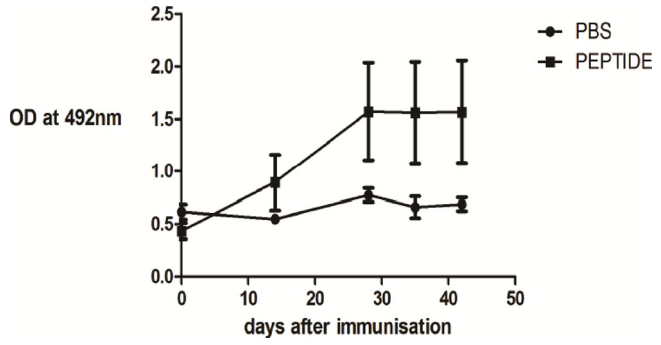

Fig. 7. Immunogenicity of the predicted epitope "FYIIPRSQES".

(Zeller et al., 2012).

Given the extreme diversity of the virus and the increasing number of viral mutations, vaccine development against rotavirus is now focused on highly specific antigenic segments of the virus which would induce neutralizing antibodies. It has been suggested that a specific evolutionarily conserved segment on the surface glycoproteins of the virus can act as an epitope or candidate of antibody recognition and that this portion could be used to develop a vaccine (Ekier et al., 2009; Ghosh et al., 2012). Recent studies suggest the distribution of conserved specific epitopes in the VP8* subunit of VP4 (Larralde and Gorzigli, 1992). It has been reported that antibodies present in the serum from infected children react against the variable VP8* region corresponding to different P[8] human rotavirus lineages (Contreras et al., 2011) thus demonstrating the importance of VP8* in antigenicity. Several VP8*subunit vaccines developed, induce rotavirus-neutralizing antibodies (Kovacs-Nolan and Mine, 2006). A recent review by (Jiang et al., 2017) stresses the importance of VP8*-based vaccine candidates.

To our knowledge the peptide identified by us is the first conserved peptide sequence of the VP8* segment of the rotavirus designed by computational methods. Earlier use of computational methods in efforts to find conserved sequences against rotavirus proteins have yielded rotavirus VP7 surface accessible conserved regions (Ghosh et al., 2012), a potential peptide region from the outer capsid protein VP4 (Hossain et al., 2016) and selective conserved motifs of VP4 and VP6 proteins for construction of a model peptide (Jafarpour et al., 2015). In our effort to find a conserved region which would help in development of a vaccine which would be effective across the globe, the conserved peptide sequences were identified in the human rotavirus A which is known to be the most common cause of acute gastroenteritis in young children (Esona and Gautam, 2015). Furthermore, as all the 390 fasta formatted sequences of the human rotavirus A VP4 protein submitted to NCBI and UniProtKB till date were retrieved for the study, it is expected that the peptide sequence if developed into a vaccine would be effective for a vast population across different countries.

The peptide expresses both B and T cell epitopes. To be a potent epitope, the peptide must be well conserved, should have good T and B cell processivity as well as have good binding affinity to maximum number of MHC alleles. A potent B cell epitope would interact with B lymphocytes and initiate specific antibody production, thus useful in vaccine development. Both B cell and T cell immune responses are important when peptide sequences are used in vaccines (Kovacs-Nolan and Mine, 2006). T cell epitope content is an important cofactor responsible for antigenicity (Weber et al., 2009). Promiscuous immunogenic T cell epitopes can bind with maximum number of HLA alleles. Hence, a vaccine constituted with these promiscuous epitopes would cover the genetic heterogeneity in the human population (De Groot et al., 2002; Lund et al., 2004). Vaccine designed using the conserved promiscuous epitopes has been reported to elicit broader T cell response against different pathogens (Ribeiro et al., 2010; Junker et al., 2012; Panigada et al., 2002).

The truncated VP8* subunit parenteral vaccines developed till date have used extraneous T cell epitope to enhance the antibody response. Residues 830–844 (P2) of tetanus toxin which represents a universal T-cell epitope, have been incorporated into a recombinant peptide VP8* subunit antigen construct (Kovacs-Nolan and Mine, 2006). More recently, the immunogenicity of another truncated VP8*subunit vaccine has been enhanced by incorporating the universal tetanus toxoid CD4 + T cell epitope P2 (Wen et al., 2014). However, a word of caution has been advocated regarding use of vaccines and biologic proteins in regard to their immunogenicity. It is important to keep in mind danger signals to the development of T effector response to autologous proteins as many protein therapeutical products are known to contain such signals (Esona and Gautam, 2015). T cell epitopes can be now predicted using *in silico* tools (Weber et al., 2009). We chose the peptide sequences which were common as both B cell and T cell epitopes, thus obviating the need to incorporate any extraneous T cell epitope. When considering B-cell antigens as potential subunit vaccines, it may be important to consider their T-cell epitope content as well since the quality and kinetics of the antibody response is dependent upon the presence of T help (He et al., 2010). Through our studies, we found the SEQ1 amino acid region as a desired peptide, as this region was found to be common for both T and B cell epitope. The best epitope was determined using Immune Epitope Database (IEDB) and Propred epitope prediction tools. ProPred gives a prediction of the MHC Class-II binding regions in an antigen sequence, using quantitative matrices derived from published literature. It also assists in selecting vaccine candidates by locating promiscuous binding regions.

A significant factor in determination of T cell immunogenicity is the binding strength of the T cell epitope to major histocompatibility complex (MHC or HLA) (Franco et al., 2006). Epitopes with higher binding affinities are more likely to be seen on the cell surface (in context of MHC molecules) where they are recognized by their corresponding T cell receptor (De Groot and Martin, 2009). The epitope SEQ1 10 mer was docked to test the interaction with the binding cleft of one of the most important and prevalent class II MHC molecules HLA-DRB1 and DRB3 with higher binding affinity (Rosetta energy). SEQ1 has nearly similar binding energy of the simulations of HLA alleles (modelled/experimental structures) which indicates that the predicted epitope is suitable for *in vivo* studies.

The binding of flexible peptides binding to HLAs play a key role in facilitating immune responses. In this study, HLA and epitope binding were predicted by CABS-dock and subsequently refined using FlexPrepDock. To address the challenge of the considerable conformational flexibility of the peptides, we have employed FlexPepDock tool for refinement of the top ranked HLA-epitope complexes obtained from CABS-dock. Recent studies have shown that FlexPepDock can produce high-quality models of epitope-HLA complexes which can be widely applied to peptide-HLA modeling and mechanism research of peptide-mediated immune responses.

The sequence SEQ1 was also found to be non-allergenic and hence suitable for parenteral administration. Allergenicity was assessed using AllerHunter server which predicts allergenicity by integrating Food and Agriculture Organization (FAO)/World Health Organization (WHO) allergenicity evaluation scheme and support vector machines (SVM)-pairwise sequence similarity. AllerHunter predicts both allergens as well as non-allergens with high specificity and hence useful for allergen cross-reactivity prediction as well (Liao and Noble, 2003; Chen and Wong, 2005). Additionally, the PIR Peptide Match does show the sequence to be only present in VP4.

Presentation of peptides by Antigen presenting cells (APCs) greatly depends on the form in which they are offered to APCs. It has been found that long peptide vaccination ultimately leads to enhanced CD8C T cell activation compared to whole protein (Rosalia et al., 2013). The peptide we have designed has a length of 10 amino acids. The recombinant peptide antigen constructs of the VP8* subunit protein of human rotavirus described by Kovacs and Nolan are also of the same length. Peptides of 20 amino acids or longer, have the advantage of

requiring processing by professional APCs, thereby reducing the chance of inducing tolerance by peptide vaccination (Bijker et al., 2008). However, synthesis and purification of longer sequences is difficult and purity of the peptide obtained is crude. Synthesis of the identified short peptide sequence in this study was easy and the protein was found to be pure.

Initial investigation into the immunogenicity of the VP8* peptide does show evidence of the peptide being immunogenic. With the recent evidence of VP8* antigens in various large, polyvalent protein complexes (Jiang et al., 2017) incorporating an increase in number of copies of the peptide for immunization as suggested in earlier reports is expected to increase the immune response to the peptide (Kovacs-Nolan and Mine, 2006). Furthermore, the vast diversity of the rotavirus with increasingly emerging mutations prompts a search for more immunogenic regions which could lead to identification of different peptides which again could form the basis of designing of a more immunogenic combined peptide vaccine.

5. Conclusion

We have identified a highly conserved peptide SEQ1 (FYIIPRSQESK) possessing both T and B cell processivity and antigenicity. It is expected to initiate good T and B cell responses and is also non-allergenic. The initial evidence of immunogenicity study suggests that the peptide could be a useful candidate for development of a parenteral vaccine against rotavirus infection. Our observations also suggest that it would be worthwhile to explore within the VP8* conserved segments, for other peptide regions which can elicit significantly high neutralizing antibodies and be used to develop new vaccines against rotavirus infection.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.06.306.

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