

In silico Multi Subunit Vaccine Design Referring Spike Glycoprotein of SARS-COV-2 (COVID-19): The World Pandemic

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Bhardwaj *et al.*: *In silico* Vaccine Design for SARS-COV-2

Contagious human coronavirus belong to family Coronaviridae and infects human respiratory system causing the disease known as COVID-19 (World Health Organization). To eradicate the severe acute respiratory syndrome coronavirus 2 pandemic, an effective vaccine should be developed. In the current study immunoinformatics procedures were employed to introduce a novel multi-epitope subunit vaccine. This multi-epitope vaccine can activate equally class I and II human leukocyte antigen and antibody mediated immune responses. Spike glycoprotein (protein data bank Id: 6VSB) of the coronavirus was selected and analysed using immune epitope database server for prediction of potential immunogenic B and T-cell epitopes. Population conservation studies of the selected protein and predicted epitopes were also performed using population conservancy analysis tool of immune epitope database resource. The two dimensional and three dimensional structure of multi-epitope vaccine were predicted and authenticated using PROCheck and Raptor-X servers. Docking results of the multi-epitope vaccine peptide with human leukocyte antigen class I and II alleles predicted efficient binding and the resulted docked models were stable during simulation. *In silico* immune evaluation using C-ImmSim server showed that the peptide could concurrently elicit cell-mediated and humoral immune responses. Immune simulation studies significantly anticipated high levels of Immunoglobulin M and Immunoglobulin M, T-helper, T-cytotoxic cells, Interferon- γ .

Key words: Epitopes, coronavirus, immunoinformatics, bioinformatics, vaccine, docking, COVID-19, SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or Coronavirus disease (COVID-19) is a novel zoonotic virus responsible for outbreak of respiratory illness, which has spread to several countries around the world^[1]. The virus was first discovered in Wuhan, China in a 41 y man on 26 December 2019^[2]. COVID-19 rapidly triggered a global health emergency, affecting 212 countries by 29 April 2020^[3]. Coronaviruses are the members of order Nidovirales, family Coronaviridae and genus β -coronavirus^[4]. A total of five major Open Reading Frames (ORF) were found in COVID-19: the ORF 1a/b region coding replicase and other enzymes, the spike or S protein ORF, M or membrane glycoprotein ORF, E or small membrane protein ORF and the N or nucleocapsid ORF (fig. 1)^[5]. Its genome consist of single-stranded plus sense RNA which is capped at 5' end and polyadenylated at 3' end^[6,7]. Contagious human coronavirus (SARS-CoV-2) binds to the receptors of

human host cells by transmembrane spike glycoprotein that makes homotrimers protruding from viral surface region^[8,9,10]. Spike glycoprotein has two subunits, S1-responsible for its binding to host cell membrane receptors and S2-responsible for the fusion of viral and host cellular membrane^[8,11]. SARS-CoV-2 uses angiotensin converting enzyme-2 (ACE-2) receptor, for entry in human host^[11,12]. Serine protease present on cell membrane, is used by SARS-CoV-2 for associating spike protein to enable its fusion with host cells^[12-14]. At present no specific drug or vaccine is available for its cure. With an objective of a potent *in silico* multi-epitope vaccine for COVID-19, was designed in this

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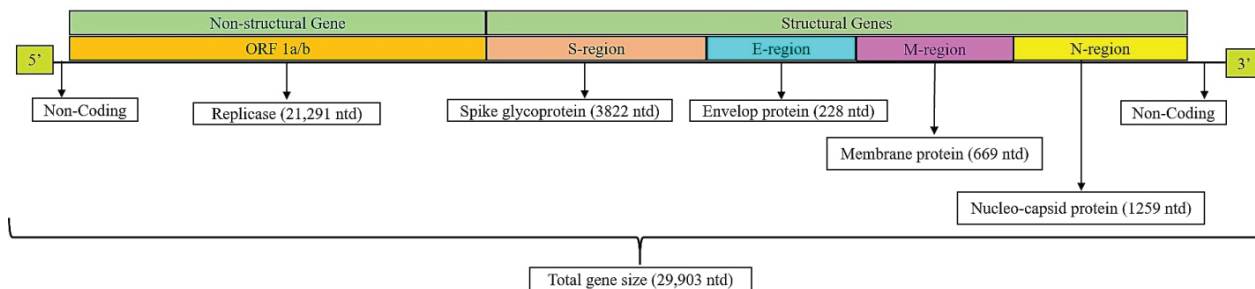


Fig. 1: COVID-19 Gene organisation showing major ORF regions, with structural and non-structural genes^[2].

research article. The initial stair of vaccine development process is selecting those antigens that can be used as immunogens. The spike glycoprotein sequence was retrieved from protein data bank (PDB) database. The S1 subunit (PDB I.D-6VSB) of the spike protein being reported to acts as receptor binding domain (RBD), binding to ACE-2 receptor with higher affinity. The T and B cell epitopes were predicted for the S1 subunit protein sequence using Immune Epitope Database (IEDB) server^[15].

MATERIALS AND METHODS

IEDB server was used to select CD8+ T cell (Tc), CD4+ T cell (Th), and B cell epitopes, in accordance with sequence conservation measures covering majorly affected countries population. For the prediction of class I and II HLA (Human Leukocyte Antigen) epitopes Tepitool tool of IEDB server was used. For prediction of B cell epitopes Discotop 1.1 tool of IEDB server was used. All the epitopes selected were validated for their antigenicity and allergenicity by Vaxijen and Allergen FP server, respectively^[16]. Further by using these epitopes and an adjuvant the subunit vaccine was designed and evaluated employing.

The subunit vaccine model was constructed and its two dimensional (2D) and three dimensional (3D) structures were predicted and validated using PSIPred, Raptor-X, Procheck 3.5 and Pro-SA web servers respectively^[17-21]. The docking studies was performed using CB-dock and HPEPDOCK docking servers to understand the binding capacity between constructed vaccine peptide (ligand) and its protein (receptor)^[22-24]. Immune simulation studies were conducted using C-ImmSim server to understand that the multi-epitope vaccine model could elicit IgG and IgM response with CD8+ and CD4+ and T cell mediated immune responses^[25]. For the designed vaccine model *in silico* expression studies on *E.coli* K12 strain was also performed using SnapGene tool^[26].

Immune adjuvants are also used which are unique in their activity and is a key requirement in vaccine

formulation. Alhydrogel (Adjuvant I.d-VO-0001241) retrieved from the Vaxjo server was used as an adjuvant for the immune interaction. Alhydrogel is a typical formulation of aluminium hydroxide (Al(OH)₃) gels, for immunological investigations^[27]. The utilization of aluminium adjuvants is linked with incitement of IL-4 and Th cells, with improved IgG1 and IgE formation^[28]. Regarding safety purpose also, Alhydrogel has substantial safety record when used with other recombinant proteins and well tolerated by humans, generating considerable immune responses^[29].

Other adjuvant Matrix-M trigger robust immune responses by activating both Th and Tc cell types with low antigen dosages and have a lower risk for allergic responses. Matrix-M is made by saponin, phospholipid and synthetic cholesterol. It can be voluntarily formulated with variety of vaccine and is well-tolerated by humans^[30]. The Matrix-M has already been used to cure Ebola pandemic^[31,32].

For docking study CB-Dock (<http://cao.labshare.cn/cb-dock/>) and HPEPDOCK servers were used. The CB-Dock server forecasts binding sites of provided protein and calculate the centers and sizes using curvature-based cavity detection method and completes docking using Autodock Vina^[33]. The CB-Dock prediction server algorithms were sensibly improved and attained >70 % success rate and also gave an interactive 3D visualization of results. The CB-Dock also perform blind docking at predicted sites, instead of whole surface of protein. The HPEPDOCK server executes blind protein-ligand docking using hierarchical algorithm^[34,35]. HPEPDOCK also examine the peptide (ligand) flexibility through a group of its conformations created by MODPEP program^[36,37].

The reverse transcription and optimization of codon was performed using Java Codon Adaptation Tool (JCat) server to express the multi-epitope vaccine construct in an expression vector^[38]. Codon advancement (optimization) was executed to express the finalized vaccine construct in the *E. coli* K12 strain (host). The

codon sequence was optimized by using additional options like, avoiding rho-independent transcription termination, restriction enzymes cleavage sites and prokaryote ribosome binding site. The conceptual methodology employed in designing multi-epitope subunit vaccine for SARS-CoV-2 is given in fig. 2.

RESULTS AND DISCUSSION

The complete protein sequences of SARS-CoV-2 (Genbank Accession no. YP_009724390.1) was obtained from PDB (6VSB). The sequence were then

subjected to epitope prediction for B-cell and T-cell using IEDB server.

Spike structural glycoprotein of SARS-CoV-2 was analysed for identification of possible human leukocyte antigen (HLA) class-I binding T cell epitopes using Tepitool tool of IEDB server^[39,40]. The sequence of spike glycoprotein was screened against 27 most frequent A and B alleles (A*01:01, A*02:01, A*02:03, A*02:06, A*03:01, A*11:01, A*23:01, A*24:02, A*26:01, A*30:01, A*30:02, A*31:01, A*32:01, A*33:01, A*68:01, A*68:02, B*07:02, B*08:01, B*15:01,

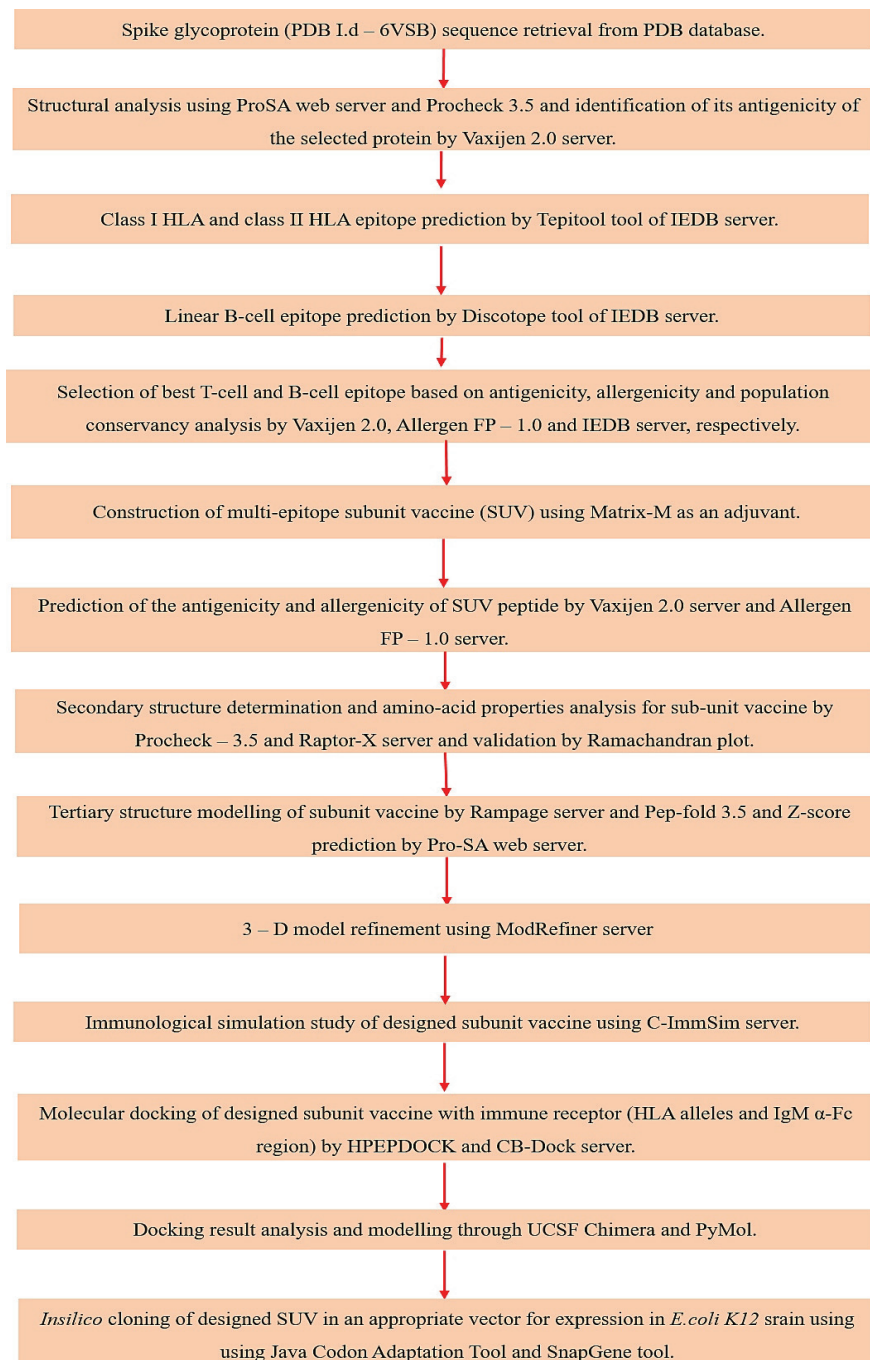


Fig. 2: Flowchart of immuno informatics top down approach employed in protein sequence based binding prediction study of class I MHC, class II MHC and B-cell for COVID-19 subunit vaccine designing.

B*35:01, B*40:01, B*44:02, B*44:03, B*51:01, B*53:01, B*57:01, B*58:01) of class-I HLA. The parameters were customized for removing duplicate peptides, synthesizing 9-mer oligopeptide sequences. The peptides were carefully chosen based on their percentile score and 100 % population conservancy (Table 1). Among 323, 9-mer oligopeptides obtained, a total of 11 potent oligopeptides were selected (Table 2) based on their allergenicity and antigenicity. The allergenicity of selected epitopes were cross-checked by AllerCatPro 1.7 and two HLA class I epitopes found allergic, was discarded. Finally, 9 potent peptides binding to class I HLA was selected for further study.

The Tepitool tool of IEDB server was used to predict peptides binding to HLA class II^[39,40]. Spike glycoprotein sequence obtained from PDB was screened for the panel of 26 most frequent alleles for promiscuous binding (HLA-DPA1*01/DPB1*04:01, HLA-DPA1*01:03/

TABLE 1: POPULATION COVERAGE CALCULATION RESULT OBTAINED BY COMBINING BOTH MHC CLASSES

Population/Area	MHC Class Combined		
	Population coverage	Average no. of hits	PC(90)
Brazil	100.0 %	24.78	15.38
China	99.97 %	21.96	13.21
Germany	100.0 %	31.32	22.32
India	99.97 %	20.11	10.0
Italy	99.97 %	26.52	15.22
North America	100.0 %	29.17	17.69
Spain	100.0 %	20.03	8.79
Average	99.99	24.84	14.66
Standard Deviation	0.01	4.09	4.26

PC is Population Coverage. MHC is Major Histocompatibility Complex.

TABLE 2: PREDICTED B-CELL AND MHC CLASS I AND II EPITOPES WITH OTHER DETAILS

Epitope code	Type of epitope	Peptide sequence	Start	End	Allergenicity score	Antigenicity score (>0.5)
B-1	B-cell	VRQIAPGQTGKIAD	407	421	Non-Allergen	1.260
B-2	B-cell	KNHTSPDVLG	1156	1167	Non-Allergen	1.403
MH-1	MHC II	GYQPYRVVLSFELL	504	518	Non-Allergen	1.074
MH-2	MHC II	PTNFTISVTTEILPV	715	729	Non-Allergen	1.134
MC-1	MHC I	DVNCTEVPV	614	622	Non-Allergen	1.239
MC-2	MHC I	FLHVTYVPA	1062	1070	Allergen/ Non-Allergen	1.334
MC-3	MHC I	FPNITNLCP	329	337	Allergen/ Non-Allergen	1.621
MC-4	MHC I	VVFLHVTVV	1060	1068	Non-Allergen	1.512
MC-5	MHC I	DIADTTDAV	568	576	Non-Allergen	1.090
MC-6	MHC I	VLSFELLHA	512	520	Non-Allergen	1.077
MC-7	MHC I	SKRVDFCGK	1037	1045	Non-Allergen	1.732
MC-8	MHC I	LEILDITPC	582	590	Allergen/ Non-Allergen	1.639
MC-9	MHC I	LPIGINITR	229	237	Non-Allergen	1.821

MHC is Major Histocompatibility Complex, which are of two types Major Histocompatibility Complex-I and Major Histocompatibility Complex-II.

DPB1*02:01, HLA-DPA1*02:01/DPB1*01:01, HLA-DPA1*02:01/DPB1*05:01, HLA-DPA1*03:01/DPB1*04:02, HLA-DQA1*01:01/DQB1*05:01, HLA-DQA1*01:02/DQB1*06:02, HLA-DQA1*03:01/DQB1*03:02, HLA-DQA1*04:01/DQB1*04:02, HLA-DQA1*05:01/DQB1*02:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DRB1*01:01, HLA-DRB1*03:01, HLA-DRB1*04:01, HLA-DRB1*04:05, HLA-DRB1*07:01, HLA-DRB1*08:02, HLA-DRB1*09:01, HLA-DRB1*11:01, HLA-DRB1*12:01, HLA-DRB1*13:02, HLA-DRB1*15:01, HLA-DRB3*01:01, HLA-DRB3*02:02, HLA-DRB4*01:01, HLA-DRB5*01:01).

The parameters were customized for removing duplicate peptides, synthesizing 15-mer peptide sequences. Among 211, 15mer peptides obtained, two potent peptides were selected based on percentile score and 100 % population conservancy (Table 2). Linear epitopes of SARS-CoV-2 spike glycoprotein for B-cell was predicted using Discotope 1.1 prediction tool of IEDB^[41,42]. The B-cell epitope prediction by Discotope tool integrates solvent-reachable surface area assessment and contact distances to predict B-cell epitope^[43]. The predicted epitopes were selected over a threshold value of -7.7 from Discotop 1.1 tool. The two selected epitopes were also analysed for their allergenicity (Allergen FP-1.0 server), antigenicity (Vaxijen 2.0 server) and population conservancy analysis (Table 1). The allergenicity of selected epitopes was cross-checked by AllerCatPro 1.7.

The selected epitopes used in designing the Subunit Vaccine (SUV) were 02 linear B-cell epitopes, 09 major histocompatibility complex (MHC) class I epitopes, and 02 MHC class II epitopes, linked by GPGPG and

AAY linkers. The Matrix-M, was opted as an adjuvant and added to the N-terminal of SUV using EAAAK linker. The different orientations of peptides were applied in SUV designing. The final vaccine peptide model of 180 amino acid residues was constructed from 13 merged epitope sequences (fig. 3).

The 2D structure and other parameters of final peptide (SUV) were obtained through PSIPred server, fig. 4. The designed sequence was also analyzed through Raptor-X property prediction tool. The Raptor-X result predicted 93 % positions of amino acids in allowed regions having alpha-helix (22 %), beta-sheet (23 %) and coiled (53 %) structure, respectively. The ramachandran (RMC) plot analysis using Procheck 3.5, resulted 94.1 % amino acid fragments are in favoured regions (A, B, L) and 5.9 % in additionally permitted regions (a, b, l, p) including glycine (4) and proline (4) residues. Based on the analysis of 118 structures of resolution (2.0 Å) and R-factor not more than 20 %, a good calibre (quality) model was expected, having more than 90 % residues in favoured regions (fig. 5).

The SUV peptide sequence generated was subjected for 3D structure modelling by PEPfold-3 and Swiss-

Model. The Ramachandran (RMC) plot to predict the validity of 3D structure of final peptide was analysed form Procheck 3.5^[44-46]. The 3D structure of final peptide was also validated through RAMPAGE and the result depicted 95.8 % residues in favoured region. The peptide of final vaccine subunit indicated decent alignment as per Z-score (fig. 6) value (reaching between 1.18 and 5.46) through ProSA-web server. The ProSA-web gave a Z-score of -2.58. The finally validated and selected model after refinement through ModRefiner server had overall quality factor of 89 %.

C-ImmSim (immune simulation) server yield reliable results with actual immune responses. The various antigen-antibody interactions are shown in fig. S1(a) (supplementary file). The primary response was depicted by significant rise in levels of IgG and IgM. The secondary and tertiary responses represented marked increments in B-cell, including levels of IgG1 and IgG2, IgM, and IgG+IgM antibodies with a corresponding decrease in antigen concentration, fig. S1(b), S1(c) (supplementary file). Similarly, high response was observed in the Th and Tc cell population with corresponding memory development (fig. S1(c)

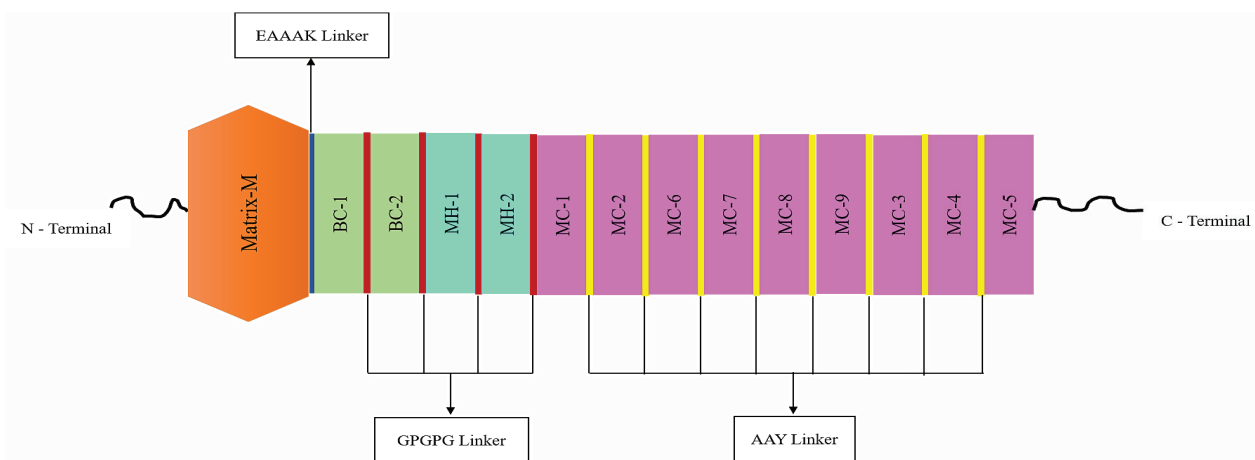


Fig. 3: Schematic presentation of the final multi-epitope vaccine peptide. The 180 amino acid long peptide sequence containing an adjuvant (orange) at N-terminal end linked with the multi-epitope sequence through an EAAAK linker (blue). B-cell epitopes and Class II MHC epitopes are linked using GPGPG linkers (Purple) while the Class I MHC epitopes are linked with the help of AAY linkers (yellow)

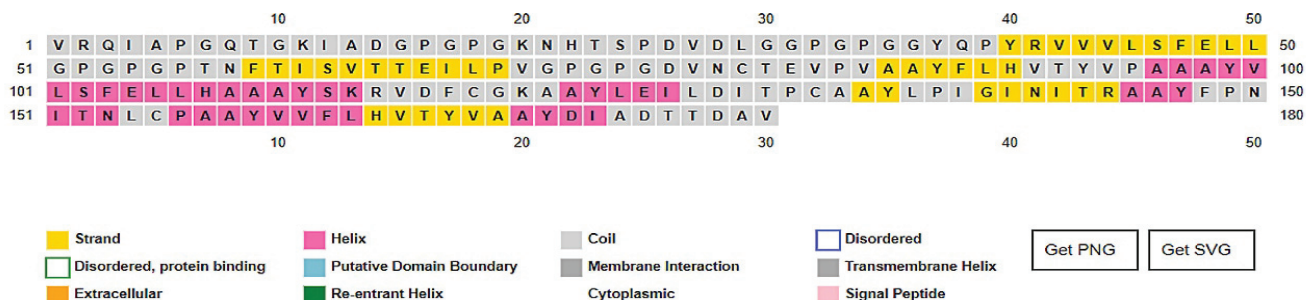


Fig. 4: Secondary structure validation with linear representation of secondary structure features of finally designed subunit vaccine.

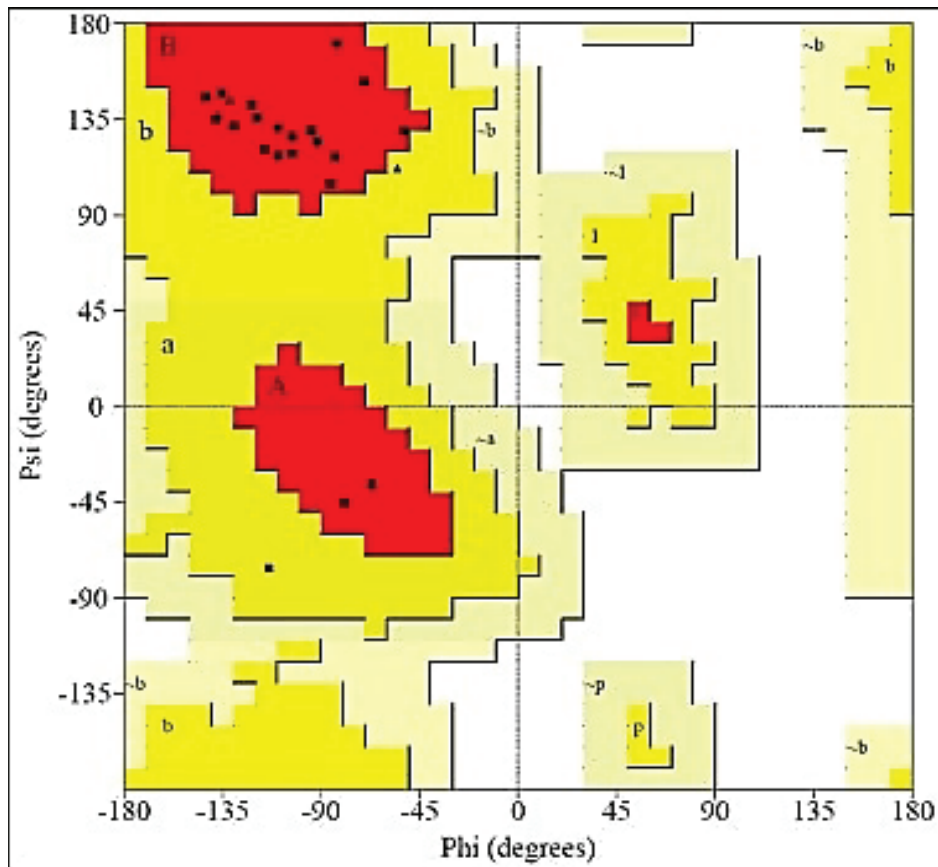


Fig. 5: The Ramachandran Plot of multi-epitope vaccine peptide retrieved from Procheck 3.5 online tool, showing 94.1 % residues in in most favored regions

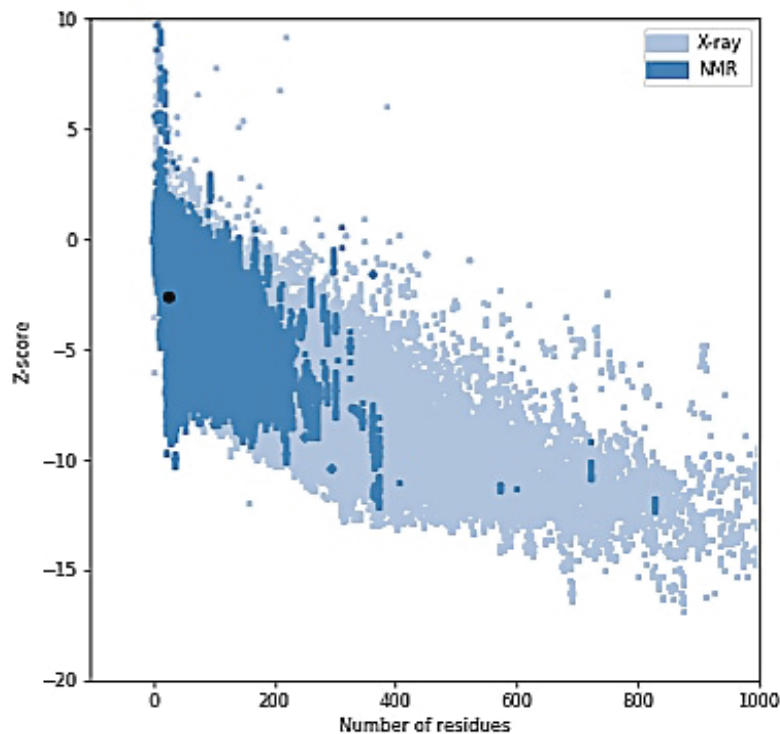


Fig. 6: Predicted z-score by ProSA web server (-2.58), for constructed SUV peptide.

and S1(d) supplementary file). The SUV peptide exposure elicited levels of IgG1, IFN- γ and Th cell concentration.

The binding affinity of SUV peptide was detected with IgM (α -Fc receptor), class I and II HLA. For docking purpose, the α -Fc receptor sequence of IgM was retrieved

from genbank (Accession No-NP_001116451.1) and secondary structure was constructed using Swiss Model online server (<https://swissmodel.expasy.org/interactive>). The constructed model was retrieved in PDB format and refined using ModRefiner tool (RMSD score-1.432 and Tm score-0.9486). The protein sequences of class I HLA (Accession No-CCH75804.1) and class II HLA (Accession No-QCF28413.1) was also retrieved from Genbank and secondary structure was constructed for docking study. The final structure of IgM (α -Fc receptor), class I HLA and class II HLA was refined by ModRefiner tool and analysed by using UCSF chimera.

The designed SUV peptide was used as ligand and IgM (α -Fc receptor) was used as receptor to perform blind docking simulation. The result of HPEPDock server depicted its top model with -182.84 docking score and the CB-Dock server gave -5.5 vina score and 401 Å cavity size volume of receptor molecule. The respective result (fig. 7) obtained was analysed by using Pymol. The docking score (binding affinity) revealed that the ligand and receptor interaction is stable enough to generate immune responses.

The ligand was blindly docked with class I HLA protein and the result depicted -235.40 docking score from HPEPDock server top model and -5.6 Vina score with 320 Å cavity size volume of receptor molecule for CB-Dock top model (Table S2, supplementary file). The respective result (fig. 8) obtained was analysed by using UCSF Chimera^[47]. The docking score revealed that the ligand and receptor interaction was stable enough to generate immune responses, activating Tc cells and release of IFN γ .

The ligand was blindly docked with class II MHC and the result depicted -286.78 docking score from HPEPDock server top model and -7.0 Vina score with 249 Å cavity size volume of receptor molecule for CB-Dock top model (Table S2, supplementary file). The respective result (fig. 9) obtained was analysed by using Pymol. The docking score revealed that the ligand and receptor interaction is stable enough to generate immune responses and develop a good response through class II MHC activating Th cells and release of interleukins.

The JCat tool resulted CAI (codon adaptation index) as 1.0 and percentage GC content as 56.66, which were used to evaluate expression level of protein^[48].

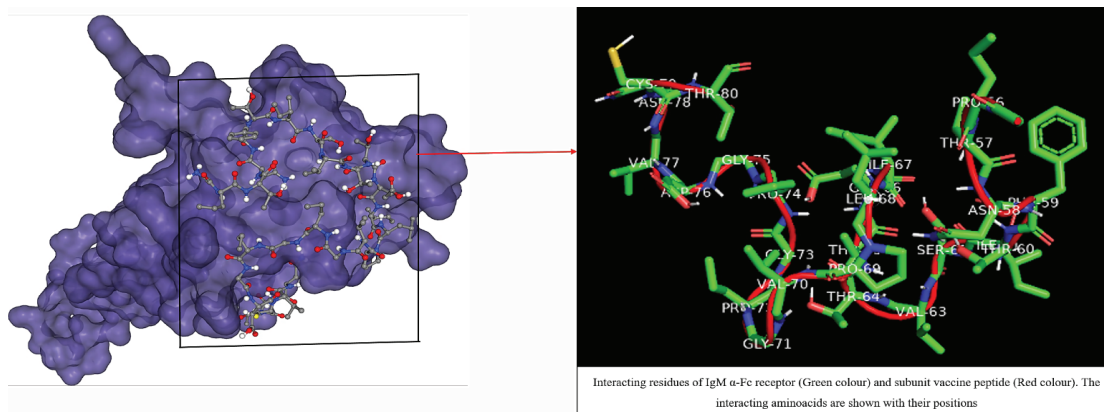


Fig. 7: Molecular docking result of IgM (α receptor) and multi-epitope designed subunit vaccine

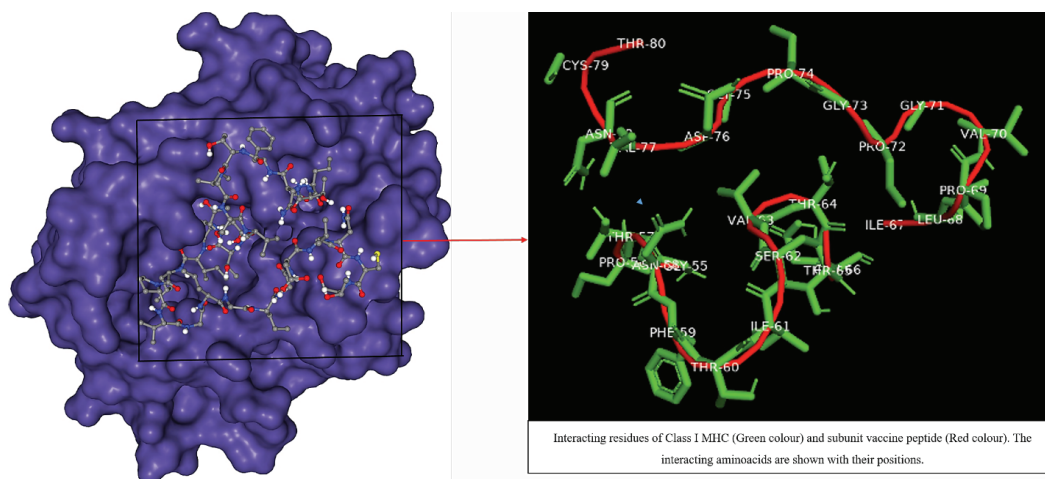


Fig. 8: Molecular docking result of class I MHC and multi-epitope designed vaccine

The CAI value of 0.8 to 1.0 indicates a good score and limits codon usage partialities. In order to clone final optimized subunit vaccine peptide construct in *E. coli*, pBluescript SK(+) vector of 2958 bp was used. Restriction sites of Bam HI and Nsp I were inserted at N and C-terminals of gene sequence, respectively. Before Nsp I restriction site a 6x histidine tag was also added to aid the purification methods. Finally, the optimized sequence was ligated into the pBluescript SK(+) vector using SnapGene tool to confirm vaccine expression (fig. 10). The size of final clone constructed was 3091 base pairs.

The COVID-19, as named by WHO (World Health Organisation), is a contagious virus which created havoc death situations around the world. More than 3.5 million affected cases and above two million deaths have occurred around the world till 30th December 2020^[49-52]. For the present research work, spike glycoprotein sequence (PDB I.d-6VSB) was retrieved from PDB database. The subsequent protein sequence was then used to identify potential epitopes (B-cell and T-cell). The B-cell activation is responsible for the outcome of humoral immune response and also the development of memory. The IgM with IgG generates

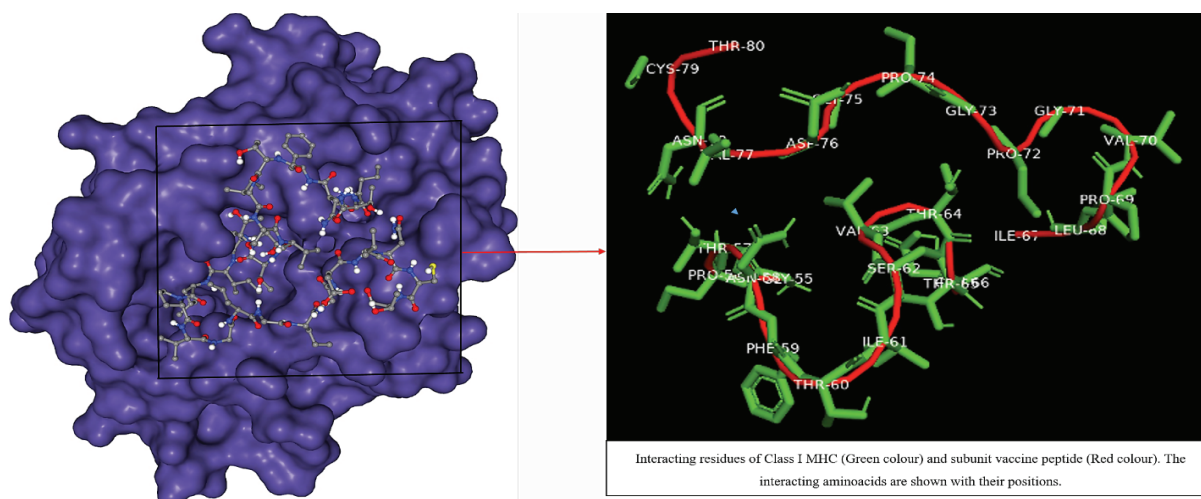


Fig. 9: Molecular docking result of class II MHC and multi-epitope designed vaccine.

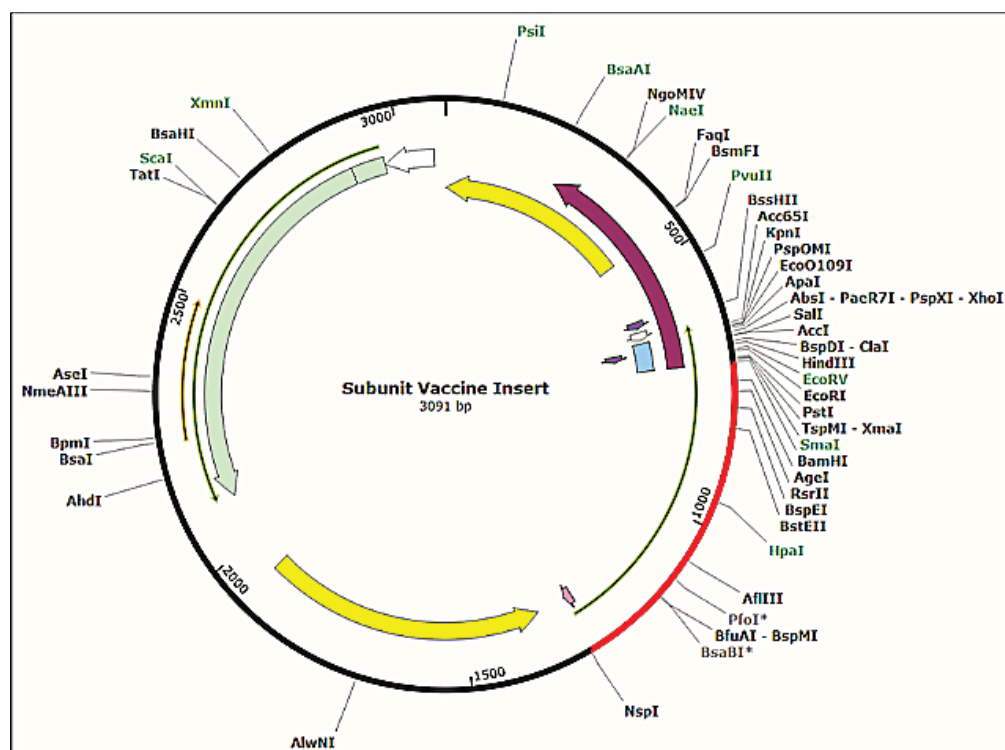


Fig. 10: *In silico* restriction cloning of the constructed Subunit vaccine into the pBluescript SK (+) expression vector where the red region indicates the gene coding for the vaccine and the black coloured circle represents the vector. A 6x his-tag is also located at Carboxy-terminal end.

humoral immune response and also acts as antigen presenting cells (APCs). The B-cell epitopes which were determined by IEDB prediction server have the potential to bring out the activation of IgG and IgM which are necessary to develop humoral response and memory cells in the body. The selected B-cell epitopes based on their propensity score was then subjected to check their allergenicity and antigenicity by Vaxijen 2.0 and Allergen FP 1.0 online server (Table 1). For prediction of T-cell epitopes for class I and II HLA Tepitool tool of IEDB server was used and epitopes were selected based on their IC50 value. The selected HLA epitopes were then identified for their allergenicity and antigenicity over a threshold value of 0.7 (Table 2). As the HLA allele differs in distribution among diverse geographical regions and ethnic clusters around the world. Population coverage analysis was performed and major population coverage (99 to 100 %) was perceived for particular 13 epitopes in majorly affected countries (Table 1).

Among the two selected adjuvants, Matrix-M is newly introduced adjuvant and reported to be used in various viral vaccine developments. It has the potential to give both B and T-cell facilitated better immune response. Now a days Matrix-M is being frequently used to design subunit multi-epitope vaccines^[49]. As per the research, Matrix-M was proved to be more antigenic than alhydrogel^[49]. The B and T-cell epitopes binding to their specified HLA alleles are shown in Table S1, supplementary file. The subunit vaccine model was constructed using class I HLA (9), class II HLA (2) and B-cell (2) epitopes by using AAY and GPGPG linkers.

The Ramachandran plot (fig. 5) depicted that 96.5 % residues were in favorable region and the Z-score predicted by ProSA server was -2.58. The Z-score validated that the vaccine peptide could be constructed and resulting better immunological response. The immune simulation results predicted that the designed vaccine model was potent enough to activate B-cell associated IgG and IgM response, among which IgG was able to develop memory of the encounter and can be preserved in the body for secondary response. The subunit vaccine was also potent to develop class I and II MHC response. The vaccine peptide also activated IFN- γ , IL-17, IL-26 and macrophages, involved in both adaptive and innate immunity.

The docking studies predicted strong binding affinities with the vaccine peptide and their receptors giving a dock score of -5.6, -7.0 and -5.5 (Table S2, supplementary file) respectively. The docking results

were further analysed by Pymol and interacting residues are depicted in fig. 7-9. The cloning studies of SUV peptide reveal its better integration (fig. 10) for the expression in *E.coli* K12 strain.

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