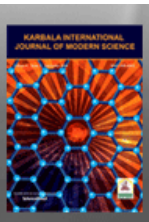

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Engineering of a Multi-Epitope Subunit Vaccine Against SASRS-CoV-2 Through the Viroinformatic Approach

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Abstract

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Keywords

Bioinformatics; SARS-CoV-2; public health; MHC-I and MHC-II; multi-epitope vaccine

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RESEARCH PAPER

Engineering of a Multi-Epitope Subunit Vaccine Against SASRS-CoV-2 Through the Viroinformatic Approach

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Abstract

The COVID-19 outbreak has infected millions of people worldwide, but no vaccine has been discovered to combat it efficiently. This research aims to design a multi-epitope vaccine using highly efficient B- and T-cell epitopes from the SARS-CoV-2 Surabaya isolate through a viroinformatic approach. First, the putative epitopes were linked together to develop tertiary structures and then docked with toll-like receptor 4 (TLR-4) that demonstrated a robust interaction with a low eigenvalue of 4.816138 e−06. Furthermore, the structure's high immunogenic response was observed and successfully cloned into the expression vector pET28a (+). This implies that the designed vaccine can prove effective in combating SARS-CoV-2.

Keywords: Bioinformatics, SARS-CoV-2, Public health, MHC-I and MHC-II, Multi-epitope vaccine

1. Introduction

At the end of December 2019, a few people from Wuhan, China, were reported to have pneumonia symptoms. Upon examination, the SARS-CoV-2 virus was revealed as the causative agent of the infection [1,2]. The novel coronavirus disease (COVID-19) pandemic was designated a public health emergency by the World Health Organization (WHO) in January 2020. It has been estimated that since 30 December 2019, over 218.94 million people have been infected with COVID-19. Moreover, about 4.53 million people have died [3]. SARS-CoV-2 has a novel characteristic of spreading rapidly since many of its patients remain asymptomatic [4,5], and diagnostic methods take time [6,7]. The genome of SARS-CoV-2, like those of other coronaviruses, encodes for a variety of structural proteins. In the genome, the membrane "M," the nucleocapsid "N," the spike "S," and the envelope "E" proteins are found as structural proteins. Additionally, non-structural proteins such as ORF1ab, ORF6, ORF3a, ORF8, ORF7a, and ORF10 are also evident [8]. The aminoacids-based genomic similarity was 76% among both SARS-CoV and

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SARS-CoV-2 [9,10]. Owing to the high degree of sequence similarity, we can use primary data of SARS-CoV on protective immune responses for developing a SARS-CoV-2 vaccine [11–14]. Cellular and humoral responses are critical host defenses against SARS-CoV. Experimentally, antibodies developed against the "S" and "N" proteins were reported to protect mice from the pathogenicity of SARS-CoV infection. Moreover, identical antibodies were discovered in SARS-CoV-2 and SARS-CoV-affected people [15–20]. On the other hand, antibody responses to the S protein were undetectable six years after recovery [21]. Furthermore, stronger antibody titers against the virus infection have been identified in more severe clinical cases of viral infection, implying that a strong antibody response alone may not be enough to suppress SARS-CoV and SARS-CoV-2 infections [22–25]. Due to the high demand for safe and effective therapies against SARS-CoV-2 [26–28]. Undoubtedly, any vaccine-based measures could be highly beneficial in the event of outbreaks or seasonal re-emergence, largely dependent on long-term protective evolution. Given their genetic similarities, recent success in developing vaccines against "SARS-CoV-1″ and "MERS-CoV" might be a significant feature in designing a vaccine against SARS-CoV-2 [29–33]. In an outbreak crisis, traditional vaccination techniques based on laboratory trials could not address the immediate needs; therefore, several therapeutic substances are being evaluated [34–37]. A bioinformatics study is a powerful tool for sorting, organizing, and processing enormous amounts of data from several research studies to build a broad immunology platform in a short period. Due to the availability of the virus genome and protein sequences, in silico analysis might be incorporated to anticipate the reported epitopes and virus features, considerably speeding up vaccine development [38–42]. The current study aimed to predict B- and T-cell epitopes from the SARS-CoV-2 M, N, and S proteins and design a multi-epitope immunogenic SARS-CoV's-2 subunit vaccine candidate using bioinformatic techniques Fig. 1.

2. Material and methods

2.1. Ethical issues

Ethical approval for the current study was obtained from the Institutional Review Board of the Dr Soetomo General Hospital, Surabaya under IRB No.IRB00008635. The Ethical Clearance from the same body was additionally obtained under No. 0099/LOE/301.4.2/VIII/2020.

2.2. Retrieval of the whole genome sequence and translation into amino acids

The SARS-CoV-2 Surabaya isolate of the Research Center for Vaccine Technology and Development, Institute of Tropical Diseases (RCVTD-ITD), under Accession No 1366505, was retrieved from the database of GISAID EpiCoV: ("https://www.gisaid.org/") for the formulation of a putative SARS-CoV-2 vaccine. The Amino acid (protein) sequence was deduced from the retrieved whole genome sequence (RNA) sequences, using the ExPaSy tool: (http://expasy.org/tools/dna.html). Protein segments were identified using the NCBI's (National Center for Biotechnology Information) Protein BLAST [43,44].

2.3. B-cell and T-cell epitopes

B-cell epitopes are particular antigen region that interacts strongly with B lymphocytes. As a result, B-cells developed antigen-specific antibodies and memory cells. The N, M, and S protein segments were fed to the "Immune Epitope Database" (IEDB) webserver (https://tools.iedb.org/bcell/) for prediction of the linear B-cell epitopes using the default criteria [45,46].

MHC, e.g., (Major histocompatibility complex) molecules are expressed on the cell surface and deliver peptides to T cells, making them essential in forming T-cell immune responses. MHC molecules are divided into two types: MHC Class-I and MHC Class-II. Furthermore, the N, M, and S protein segments were loaded into the IEDB's MHC-Class-I and MHC-Class-II binding prediction-free online server: (http://tools.iedb.org/mhc/n) for T-cell epitope prediction. We employed multiple approaches accessible on the server for T-cell prediction, including the MHC-NP net CTLpan1.1 web server [47,48] and the Rank PEP web server. However, we utilized the IEBD-recommended 09.2020 (NetMHCpan EL 4.1) results. The T-cell epitope length for humans was specified as 9-mer and 15-mer for MHC Class-I and MHC Class-II, respectively. HLA (Human Leukocyte Antigen) molecules on the cell surface give peptides that govern the interactions between T-cells and antigen-presenting cells, essential for adaptive immunity. Because there is a varied array of antigens has been discovered a high rate of recognition by the various HLA-molecules in the population, as previously described [49]. HLA-A*01, A* 26, A*03, A*11, A* 02, A* 24, A*32, and HLA-B*35, 27, 51 were utilized in this study for MHC-I. In contrast, HLA-DRB1*03, 07, 15, 13, 04, 11 were used for MHC-II.

Peptides with a percentile rank of less or equal to one (≤1) were designated as sequence epitopes [50].

2.4. Prediction of non-toxicity, non-allergenicity, and antigenicity of B-cell and T-cell epitopes

The predicted epitopes' antigenicity was tested through the online web server VaxiJen-v2.0 (https://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html), by applying the default-threshold [51]. This web server was designed to categorize antigen exclusively based on physicochemical characteristics of protein rather than sequence alignment. Allergy is a condition of hypersensitivity to generally harmless items like medicines. Allergens are minute antigens that elicit an IgE antibodies response in most people. Subsequently, in the current study, we fed our predicted epitopes into the AllerTOP-v.2.0, web: (https://www.ddg-pharmfac.net/AllerTOP/): keeping settings on default [52,53]. It is an alignment-free online web that predicts allergy-free epitopes based on the physicochemical features of proteins. Moreover, to assess the toxicity risk of predicted epitopes, these epitopes were pasted into the "ToxinPred" web-tool: (https://webs.iiitd.edu.in/raghava/toxinpred/multi submit.php-) [53,54]. In the construction of vaccines, conserved epitopes give more comprehensive protection against multi-strains than epitopes chosen from a diverse range of genomic regions. Therefore, the conservancy of B-cell epitopes was analyzed using the Conservancy-Analysis tool in the IEBD sever [55].

2.5. Analyses of population coverage and epitope conservation

Population coverage is an essential factor in vaccine development. It is more affected by distinct HLA-types present in different frequencies at different ethnicities than the MHC-polymorphism. The universal coverage of interacting epitopes of MHC Class-I and MHC Class-II alleles was carried out using the IEBD Population-Coverage server: (http://tools.iedb.org/population/-). Because of divergence in MHC-HLA allele distribution around the globe, the population-coverage of *Homo-sapiens* MHC Class-I and MHC Class-II interacting molecules was conducted. Moreover, the conservancy of the predicted epitopes was also tested using the IEDB Conservancy-Analysis tool [56].

2.6. Construction of multi-epitope subunit vaccine

A multi-epitope subunit vaccine was developed utilizing T-cell (MHC-I & MHC-II) and B-cell epitopes. For the development of the vaccine, the 50S ribosomal protein L7/L12 was employed as an adjuvant. In addition, EAAAK linkers were also utilized to connect the adjuvant with the B-cell epitope. In contrast, GPGPG (Gly-Pro-Gly-Pro-Gly) and AAY (Ala-Ala-Tyr) linkers connected the B-cell with the MHC Class-I and MHC Class-1 with MHC Class-II epitopes, respectively. Moreover, there were overlapping sections in the B-cell; therefore, MHC Class-I and MHC Class-II were merged to eliminate overlap [56].

2.7. Physio-chemical analysis of multi-epitope subunit construct

To evaluate the physicochemical characteristics of our engineered subunit vaccine, we used the ExPASY ProtParam program: (https://web.expasy.org/protparam/). The service displayed theoretical-pI, amino acid composition, aliphatic-index, instability-index, grand average of hydropathicity (GRAVY), and molecular weight of the subunit construct [57,58]. Furthermore, the solubility rate of the subunit construct was determined using the SOLpro web: (http://scratch.proteomics.ics.uci.edu./) [56].

2.8. Structure analysis, refinement, and validation

The subunit construct's secondary structure was analyzed using the online Raptor X tool: (http://raptorx.uchicago.edu/Structure Prediction/predict/) [59]. The discovery of the essential role of the protein components that constitute cellular proteomes is a fundamental issue in modern biological sciences. Therefore, developing a credible three-dimensional (3D) atomic structure (model) of proteins is critical in the current scenario. Thus the multi-epitope subunit's tertiary structure was generated using the PHYRE2 protein fold recognition server [60]. The tertiary structure was refined using the GalaxyRefine: (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE), and the RAMPAGE: (http://mordred.bioc.cam.ac.uk/rapper/rampage.php) web-server was used to validate the refined 3D design [56].

2.9. Molecular docking and molecular dynamics simulation (MDS)

The Cluspro.2.0 web server: (https://cluspro.org/home.php) was used for protein–protein docking in order to determine the interaction between the refined subunit construct and the toll-like receptor 4's (TLR-4's) ligand-binding domains (LBDs) [61]. Furthermore, the surfactant protein A (1R13; carbohydrate

recognition and neck domains) was used as a control (C4) during the docking procedure with the TLR-4 receptor [62]. Furthermore, the docking results of vaccine + TLR-4 complex were evaluated for protein–protein interaction in the PDBsum website (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html) [63]. The iMODS web server (http://imods.chaconlab.org/) was used to perform MDSs for critical component constructs analysis by altering the formed complex's force field concerning various time intervals [64].

2.10. Codon optimization and in silico method for peptide expression

We used the following online weblink to predict the host system for our designed vaccine: http://expsys.weizmann.ac.il/expsysb/suggestES. The Java Codon Adaptation Tool (JCat) was used for codon optimization, expression creation, and reverse translation [65]. The optimization process was utilized to create a vaccine using *Escherichia coli* as the host organism (strain K12). Rho-independent transcription, ribosomal binding sites, and restriction enzymatic cleavage sites were chosen as additional options [66]. The in silico cloning was done with the *E.coli* pET-28 (+) expression vector. The nucleotide sequence was obtained from the Addgene vector database [67]. SnapGene v3.2.1 (GSL Biotech LLC, California, U.S.A.) was used to clone poly-epitope subunit vaccines [66–68].

2.11. Immune stimulation of the engineered construct

The host's immune response to the vaccine was propagated through the online C-ImmSim web server. This web server predicts the humoral and cellular responses to a particular antigen. According to the vaccine's preventative strategy, we planned three consecutive doses on days 1, 28, and 56 [62]. The simulation steps were fixed at 1050, and the simulation volume was set to default [69].

3. Results

3.1. Retrieval of whole-genome sequence and translation into amino acids

In the current study, the SARS-CoV-2 Surabaya isolate of the RCVTD-ITD, with accession No. 1366505, was retrieved from the GISAID EpiCoV database: (https://www.gisaid.org/) on 27 July 2021 for the construction of multi-epitope subunit vaccine. The whole-genome sequence was translated into protein using the ExPASY tool (https://web.expasy.org/translate/). Segments from three types of proteins—S, M, and N—were selected from the translated amino acid sequence. Confirmatory identification was performed through the NCBI's Protein BLAST tool. The chosen segments of proteins S, M, and N comprised 577 amino acids (start position = 7847), 235 amino acids (start position = 9572), and 222 amino acids (form position = 8805), respectively.

3.2. Prediction and selection of B-cell epitopes

A total of 34 B-cell epitopes with a threshold score of 0.5 were predicted on N (epitopes = 8), M (epitopes = 6), and S (epitopes = 20) protein sequence. In this study, the predicted linear epitopes in BepiPred depicted the scores—average: 0.454, 0.435, and 0.532; minimum: 0.185, 0.245, and 0.228; maximum: 0.631, 0.668, and 0.709 on S, M, and N proteins, respectively. The epitopes were fed to the antigenicity prediction webserver: VaxiJen v2.0 using a default value of the threshold and 7 B-cell antigenic epitopes. The antigenic score for these potential B-cell candidates ranged from 0.4001 to 0.5859. These predicted antigenic linear B-cell epitopes were analyzed based on non-allergenicity and non-toxicity. Five B-cell epitopes were potential candidates for subunit vaccine formulation (Table 1). The conservancy analysis of the finally antigenic, non-toxic, and non-allergenic B-cell epitopes was performed using the IEBD Conservancy Analysis tool; the epitopes were highly conserved with 100% coverage and identity conservation.

3.3. T-cell epitope prediction

3.3.1. MHC Class-I and MHC Class–II binding prediction profile

This study applied T-cells of 9 (nine) mer and 15 (fifteen) mer lengths for MHC Class-I and MHC Class-II, respectively. The IEBD tool was used for MHC-I and MHC-II binding prediction of N, M, and S proteins. This study applied T-cells of 9 (nine) mer and 15 (fifteen) mer lengths for MHC Class-I and MHC Class-II, respectively. Consequently, a total of 10,277 epitopes were found in MHC-I: S (epitopes = 5752), M (epitopes = 2184), and N (epitopes = 2341). Moreover, a total of 4356 epitopes were found in MHC-II: S (epitopes = 2454), M (epitopes = 912), and N (epitopes = 990). We chose epitopes with high MCH-I and MCH–II binding affinity and a percentile rank of $\leq$1. Finally, seven highly affinitive, antigenic, non-allergenic, and non-toxic MHC-I epitopes (Table 2) and 10 MHC-II epitopes with the same properties (Table 3) were

Table 1. B-cell epitopes prediction in Surabaya isolate.

Protein name	Peptide	antigenic	Non allergic	Non toxic
Nucleocapsid	LKEQHCQKASTQKGAEAAVKPLLVP	Yes	Yes	yes
	LLLLEWLAMAVTKKSAAEASKKPRQKRTATKA	Yes	Yes	yes
	IDAYKTFPPTEPKKDKKKKADETQALPQRQ	Yes	Yes	yes
	KKQQTVTLLPAADLDDFSK QLQ QSMSSADS			
Spike	AENSVAYSN	Yes	Yes	yes
	LPDPSKPSKRSF	Yes	Yes	yes

Table 2. Showing the highly effenitive MCH-I epitopes of Surabaya isolate.

Protein name	Peptide	Antigenicity	Non- allergic	Non-Toxic
Membrane	MACLVGLMW	yes	yes	yes
	ATSRTLSYY	yes	yes	yes
	SYFIASFRL	yes	yes	yes
Spike	IPTNFTISV	yes	yes	yes
Nucleocapsid	KTFPPTEPK	yes	yes	yes
	AQFAPSASA	yes	yes	yes
	LLLEWLAMA	yes	yes	yes

Table 3. Showing the highly effenitive MCH-II epitopes of Surabaya isolate.

Protein name	Peptide	Antigenicity	Non- allergic	Non-Toxic
Membrane	ANRNRFLYIIKLIFL	yes	yes	yes
	RNRFLYIIKLIFLWL	yes	yes	yes
Spike	AIPTNFTISVTTEIL	yes	yes	yes
	IAIPTNFTISVTTEI	yes	yes	yes
	PTNFTISVTTEILPV	yes	yes	yes
	IPTNFTISVTTEILP	yes	yes	yes
	TNFTISVTTEILPVS	yes	yes	yes
	CSNLLLQYGSFCTQL	yes	yes	yes
	SNLLLQYGSFCTQLN	yes	yes	yes
	NLLLQYGSFCTQLNR	yes	yes	yes

selected as potential candidates for the vaccine. The antigenic score was predicted as 0.4821 and 0.4812 (minimum), and 0.8820 and 1.1691 (maximum) in MHC-I and MHC-II, respectively, for those selected as potential epitopes for vaccine construct.

3.4. Analyses of population coverage and epitope conservation

The IEBD Population Coverage tool was used to determine the global coverage of interacting epitopes of MHC-I and MHC-II alleles. There is a divergence in MHC-HLA allele distribution around the globe. Therefore, the population coverage of *H. sapiens* MHC-I and MHC-II interacting molecules was conducted. The following distribution was obtained: 92.06% for Europe, 90.71% for Oceania, 88.28% for North America, 84.66% for East Asia, and 83.77% for Southeast Asia (Table 4). Moreover, country-wise coverage was found to be 91.98% for the Philippines, 90.51% for England, 88.54% for Saudi Arabia, 85.85% for Taiwan, 84.78% for France, 82.17% for South Korea, and 80.01% for Japan. Moreover, the predicted epitopes' conservancy was assessed through the IEDB Conservancy Analysis tool. It was found that all the epitopes were 100% conserved.

3.5. Construction of multi-epitope subunit vaccine

A multi-epitope subunit vaccination was developed using MHC-I, MHC-II, and B-cell epitopes. Furthermore, the 50S ribosomal protein L7/L12 was used as an adjuvant in the vaccine's development. EAAAK linkers were used to link the L7/L12 (adjuvant) to the B-cell epitope. In contrast, GPGPG and AAY linkers were used to link the B-cell to the MHC-I and MHC-II epitopes, respectively. Moreover, there were overlapping sections in B-cell; therefore, MHC-1 and MHCII also were merged to eliminate overlap. In this multi-epitope construct, a total of 7, 10, and 5 MHC-1, MHC-II, and linear B-cell epitopes, respectively, were used. The constructed multi-epitope subunit sequence had a

Table 4. Predicted population coverage of the constructed vaccine worldwide.

population/area	Class I			Class II			Class combined		
	coverage[a]	average_hit[b]	pc90[c]	coverage[a]	average_hit[b]	pc90[c]	coverage[a]	average_hit[b]	pc90[c]
Europe	83.92%	1.19	0.62	50.62%	2.04	0.41	92.06%	3.23	1.08
Oceania	86.08%	1.13	0.72	33.27%	0.89	0.3	90.71%	2.02	1.02
North America	77.43%	1.03	0.44	48.06%	1.87	0.39	88.28%	2.9	0.85
East Asia	79.48%	1.06	0.49	25.25%	0.87	0.27	84.66%	1.94	0.65
Southeast Asia	78.11%	1.0	0.46	25.83%	0.84	0.27	83.77%	1.84	0.62
South Asia	62.35%	0.79	0.27	51.33%	2.16	0.41	81.68%	2.95	0.55
Northeast Asia	75.02%	0.96	0.4	26.62%	0.94	0.27	81.67%	1.9	0.55
West Indies	68.19%	0.9	0.31	38.53%	1.43	0.33	80.45%	2.33	0.51

molecular weight of 59974.20 Da based on 563 amino acids.

3.6. Antigenicity, toxicity, and allergenicity analysis of the subunit vaccine construct

The constructed subunit vaccine sequence was subjected to the VaxiJen v 2.0 webserver to evaluate the antigenicity, which was antigenic with and without adjuvant. Then, the sequence was tested in Aller TOP v.2.0 server, and it was found that the construct was non-allergenic with and without adjuvant. The non-toxicity of the multi-epitope construct without adjuvant and of the adjuvant itself was tested. The score of 0.5059 was predicted with adjuvant, while the score without adjuvant was 0.5433.

3.7. Physio-chemical and solubility characteristics of multi-epitope subunit construct

The physical and chemical properties of the multi-epitope construct were analyzed through the ExPASY ProtParam web server. The current multi-epitope subunit construct's molecular weight was 59.97420 kDa. The theoretical pI of protein was 8.69, instability index (II) was 30.90, the aliphatic index was 89.98, and GRAVY was 0.065. The solubility rate was found to be 0.960121 when our construct was analyzed through the SOLpro web server of SCRATCH Protein Predictor.

3.8. Secondary structure of subunit construct

The secondary structure of the multi-epitope construct was analyzed through the Raptor X tool to determine the nature of the protein. This secondary protein structure analysis revealed 32% helix, 11% beta stands, and 56% coils. Moreover, a total of 62%, 19%, and 18% exposed, medium-exposed, and buried contents, respectively, were found. In the current protein structure, 39% of positions were in disordered domains.

3.9. The tertiary structure of the subunit construct

In this study, the PHYRE2 protein fold recognition server was used to develop the tertiary structure of our multi-epitope subunit construct. The top predicted model was selected based on 100% confidence and maximum coverage and identity from the 120 predicted models (Fig. 2).

3.10. Refinement process for the tertiary structure

The projected 3D model of the multi-epitope construct was submitted to the GalaxyRefine web server, and five different refined models were found. In the current study, we selected Model 4 by considering various parameters of refinement: MolProbity (2.147), RMSD (0.213), and GDT-HA (1.0000) (Fig. 2). The current model-calculated clash score was found to be 12.8, the poor rotamers score was found to be 2.2, and the Ramachandran-favored score was found to be 96.0%. In contrast, our initial model showed a MolProbity score of 1.856, RMSD of 0.000, and GDT-HA of 1.0000. The initial model-calculated clash score was found to be 13.3, the poor rotamers score was found to be 1.1, and the Ramachandran-favored score was found to be 96.8%.

3.11. Validation of refined 3D structure

The refined structure was validated through the RAMPAGE web server. The structural analysis was performed, and the Ramachandran plot was developed for the protein structure. Before refinement, 92.9% region was lying in the favorite region, 3.5% in the additional allowed regions, and 2.7% structural region was found to be in the generously allowed regions, as the refinement process lowered the critical errors of the 3D model. After refinement, the RAMPAGE-generated plot showed 96.5% residues in the favorite region, 2.7% in the additional allowed region, 0% in the generously allowed region, and 0.9% in the disallowed regions (Fig. 3).

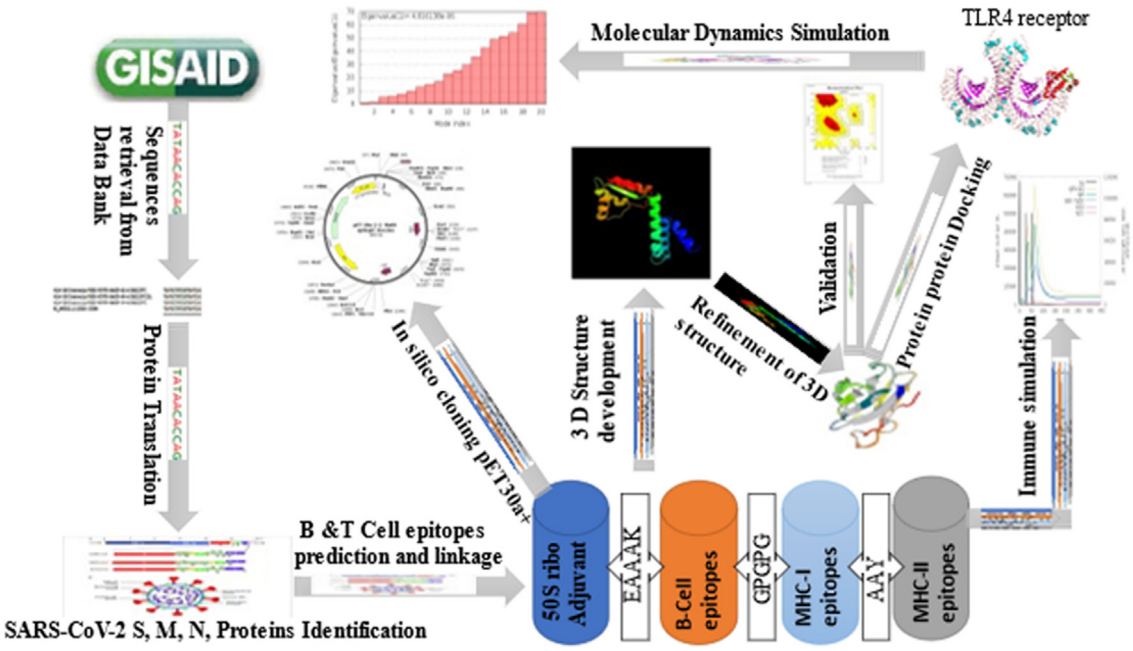

Fig. 1. Graphical abstract of the study.

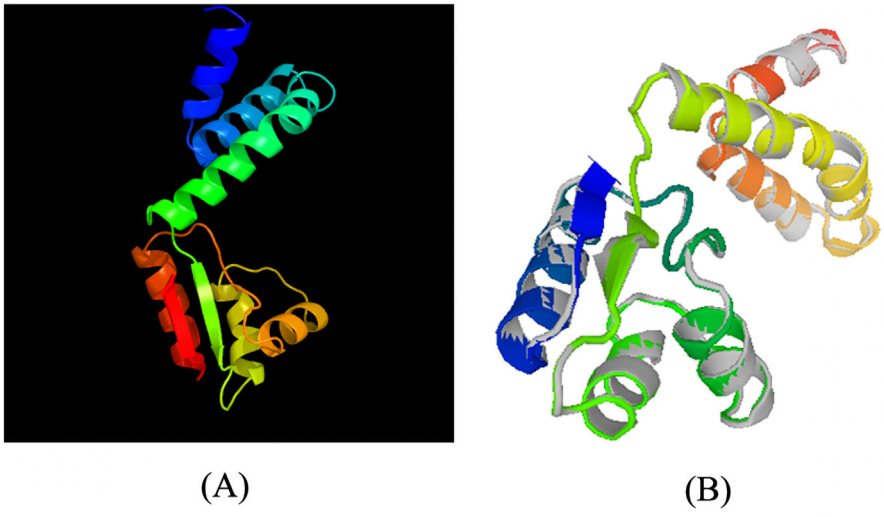

*Fig. 2. Showing the 3D Structure of Multi-epitope Construct. **(A)** is depicting the tertiary structure predicted through the PHYRE-2 Web-Serwer and **(B)** is refined 3D-structure developed by Galaxy Refine webserver.*

3.12. Molecular docking

The molecular docking of the refined vaccine Model 4 and LBD of immune receptors TLR-3 and TLR-4 (4G8A) were conducted through the protein–protein docking webserver Cluspro2.0. This docking process predicted 30 different models for the TLR-4 complex. Among all the models obtained after the analysis, we selected Model 0 of the vaccine + TLR-4 complex; it had the lowest docking energy of −753.3 kcal/mol and 92 cluster members. The PDBsum server revealed a highly stable bonding affinity between vaccine construct and TLR-4. Our vaccine design linked TLR-4 potential residues through 62 hydrogen bonds and 18 salt bridges (Fig. 4). Moreover, the complex of vaccine + TLR-4_C4 (−686.1 kcal/mol) had more incredible energy than the vaccine + TLR-4, clearly indicating that our vaccine has a more robust interaction than the control. The primary interacting

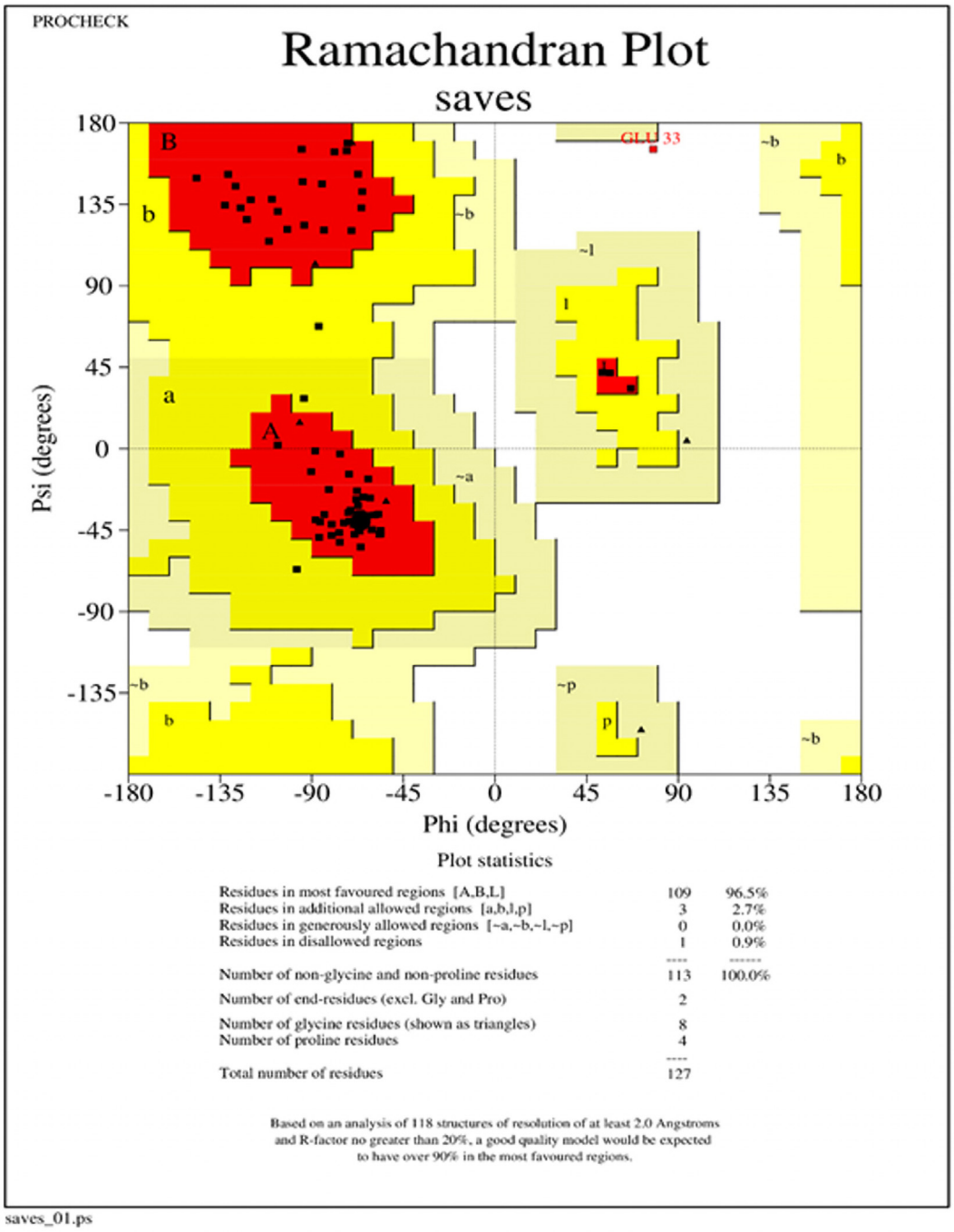

Fig. 3. Showing the validation of 3D refined structure of vaccine construct performed through RAMPAGE web server.

residues among vaccine, TLR-4, and vaccine + TLR-4_C4 are depicted in Fig. 4. It was found that the vaccine attaches to the TLR-4 through the following residues: Ser386:Ser386, Ala366:Asn365, Gly410:-ValI411, Val411:ValI411, Val411:Gly410, Phe533:-Phe533, Asn365:Ala366, His458:His458, and Gln507:Gln507. Meanwhile, the C4 control attaches to the vaccine + TLR-4 complex through the following residues: Phe553:Phe553, His458:His458, Ala366:Asn365, Val411Gly410, Val411:Val411, Gly410:Val411, Gln507:Gln507, Asn365:Ala366, and Ser386:386 (Fig. 5).

3.13. Molecular dynamics (MD) simulation

The vaccine + TLR-4 complex was fed to the iMODS web server. A normal mood analysis was performed to assess the vaccine + TLR-4 complex's

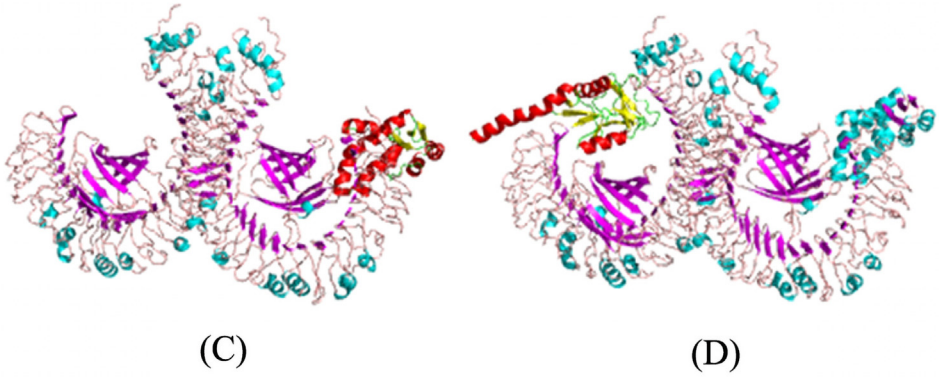

Fig. 4. Showing the molecular docking results predicted by the Cluspro webserver (C) is depicting molecular docking of multi-epitope subunit vaccine construct and receptor TLR4. (D) is showing the molecular docking of multi-epitope subunit vaccine and TLR4 complex with C4 control.

internal coordination. The complex's eigenvalue was calculated to be 4.816138 e−06 (Fig. 6 (J)). The deformability results in individual deformation of each residue, as seen by the chain hinges approach (Fig. 6 (H)). In addition, there is a gradual decrease of variance in each typical mood (Fig. 6 (K)). All of these findings point out stable binding interactions in the vaccine + TLR-4 complex.

3.14. Codon optimization and in silico method for peptide expression

JCat was used for codon optimization, expression creation, and reverse translation. The optimization process was utilized to create a vaccine using *E. coli* as the host organism (strain K12). The length of the optimization codon was 1689 bp nucleotides. The

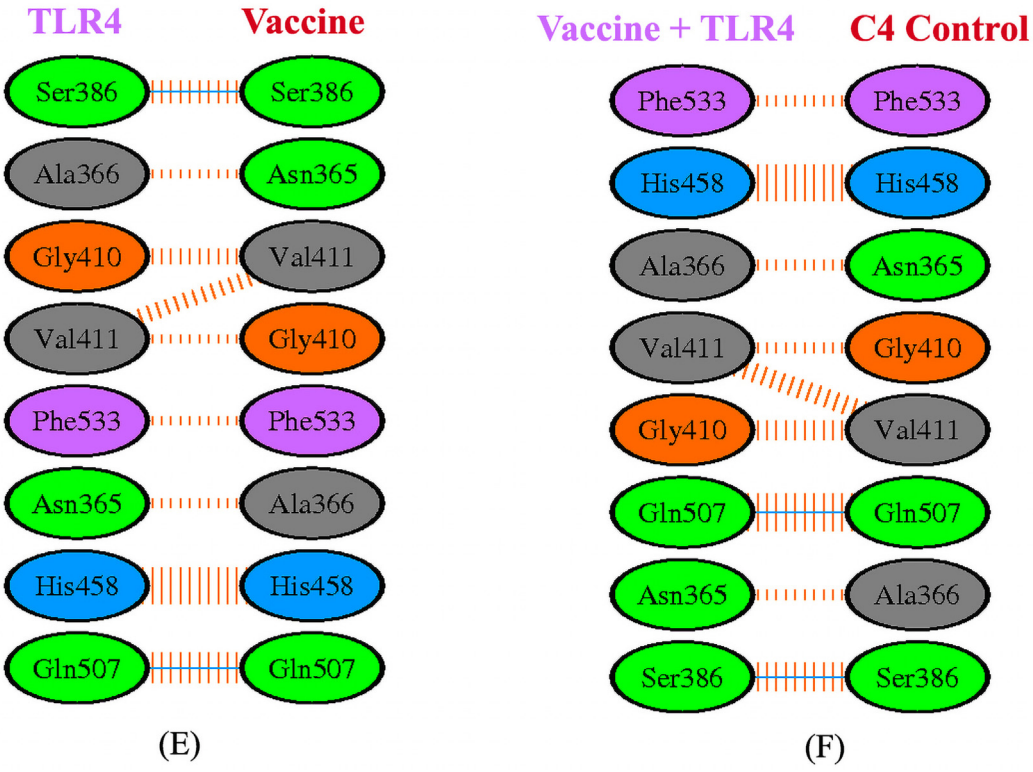

Fig. 5. Showing the interaction results developed through the PDBsum webserver. (E) is depicting the interaction between TR4 and vaccine construct. (F) is showing the TLR4+Vaccine complex binding interaction of the residues with C4 control.

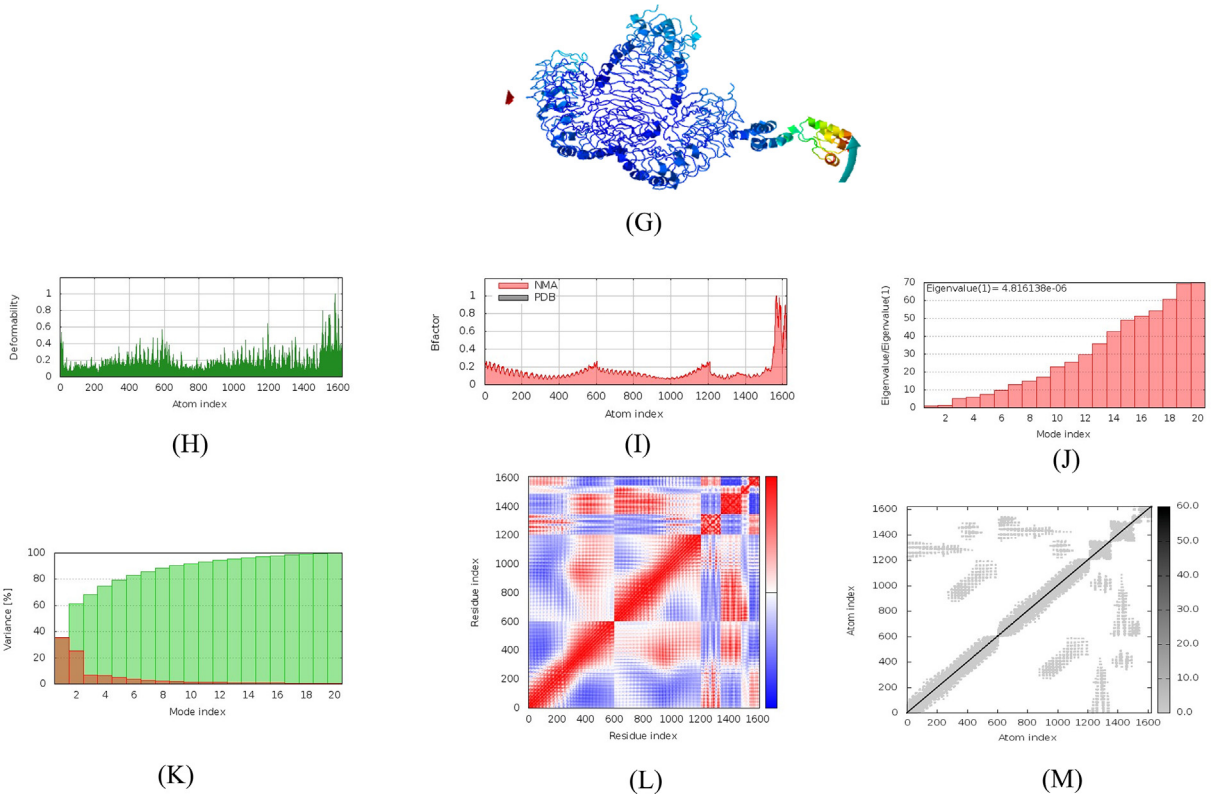

*Fig. 6. Showing the MDS results of vaccine and TLR4 docking complex. **(G)** is describing the mobility of vaccine and receptor directly towards each-other, **(H)** is describing high the deformability regions in B factor plot, **(I)** is depicting the similarity in NMA and PDB which means our actual results of complex and the simulation results are same. **(J)** is showing the low Eigenvalue of our construct as Eigenvalue is directly proportional to the deformability of construct, **(K)** is describing the individual and cumulative variance in red and green color respectively. **(L)** is depicting the co-variance plot of our construct in which co- relationship, uncorrelation and anti- correlation are described with red, white and blue colors respectively, **(M)** is describing the stiffness level of our construct in which dark gray color is showing the stiffer region.*

codon adaptation index (CAI) was found to be 0.94 for the approved sequence. In contrast, guanine-cytosine (GC) content was found to be 52.39. The in silico cloning was done with the *E. coli* pET-28a + expression vector. The nucleotide sequence was obtained from the Addgene vector database Snap Gene v3.2.1 (GSL Biotech LLC, California, U.S.A.) The codon sequence of the multi-epitope subunit construct was inserted between the HindIII (542) and NaeI (1472), which developed a clone of 4843 bp (Fig. 7).

3.15. Immune stimulation (IS) of the constructed subunit vaccine

The host's immune response to the vaccine was propagated through the online C-ImmSim web server. The simulation steps were adjusted at one thousand and fifty (1050), and the simulation volume was fixed at default. The IgM increase is the indicator for the primary response. The secondary and tertiary responses are characterized by a higher level of B-cell population and high levels of IgG1 + IgG2, IgM, and IgG + IgM. Furthermore, the current study revealed cytokine and interleukin production, which depicts the vaccine's efficiency in triggering an immune response. TGF-, IFN-, and IL-2 were also identified in significant concentrations, all of which are vital for co-stimulation of T-cell activation (Fig. 8).

4. Discussion

COVID-19 has become one of the world's most critical public health concerns. Thus, investing time to develop efficient preventive strategies is worth it. Compared to traditionally adapted approaches used in SARS-COV-2 vaccine designs, bioinformatics techniques play a significant role in easing and speeding up the prediction of potential epitope vaccines [41,60,70,71,72]. Although several studies have used immunoinformatic approaches to develop possible vaccines against SARS-CoV-2 [62,73–75], these in silico investigations for SARS-CoV-2 targeted both non-structural and structural proteins. Our study focused largely on structural

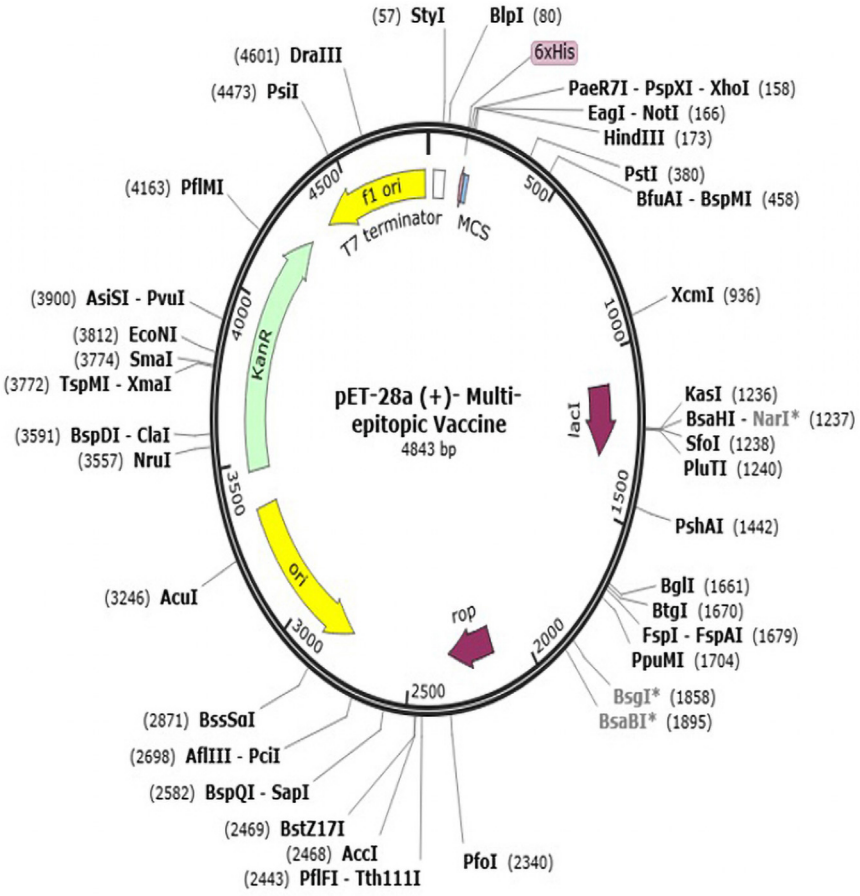

Fig. 7. Showing the insertion of multiepitope vaccine in pET-28-a(+) expression vector between HindIII and NaeI and found in results a colon of 4843bp.

proteins since they are promising for generating an effective and safe immune response against SARS-CoV-2. Furthermore, we focused on local Surabaya isolates for vaccine development in this work, as local viral vaccines are more effective and efficient against viral infections [76]. The three structural proteins of the SARS-CoV-2 virus were identified to form richly immunogenic epitopes that can trigger cellular and humoral responses. Antigenic, non-allergenic, and non-toxic B- and T-cell epitopes were created using the amino acid sequence of 1,366,505 from the Surabaya isolate. S, M, and N proteins were identified in the amino acid sequence of the SARS-CoV-2 Surabaya isolate, and B- and T-cells were produced for each of these proteins individually [50]. The sequences of the N (epitopes = 8), M (epitopes = 6), and S (epitopes = 20) proteins revealed a total of 34 B-cell epitopes. The antigenicity, non-allergenicity, and non-toxicity of these linear B-cell epitopes were determined using the VaxiJen v2.0, Aller TOP v.2.0, and ToxinPred servers respectively. We adopted this method as described in an earlier study [56], and 5 B-cell epitopes were identified as potential candidates for subunit vaccine formulation (Table 2). In this investigation, we used 9-mer and 15-mer T-cell lengths for MHC-I and MHC-II, respectively [50]. The N, M, and S proteins were used to predict MHC-I and MHC−II binding using the IEBD tool, yielding a total of 10,277 MHC-I epitopes and 4356 MHC-II epitopes. We chose epitopes with high MCH-I and MCH−II binding affinity and a percentile rank of ≤ 1 [50,56]. A total of seven highly affinitive, antigenic, non-allergenic, and non-toxic MHC-I epitopes were chosen as possible vaccination candidates (Table 3), and 10 MHC-II epitopes with the same properties were chosen (Table 4).

Once, B-cells were the only source for developing a possible vaccine. B-cell responses are significant because they are responsible for producing antibody-based immunity. MHC-I and MHC-II T-cells with HLA designs developed through bioinformatics are more faster and effective in clinical research [77]. T-cells can trigger a significant and cross-

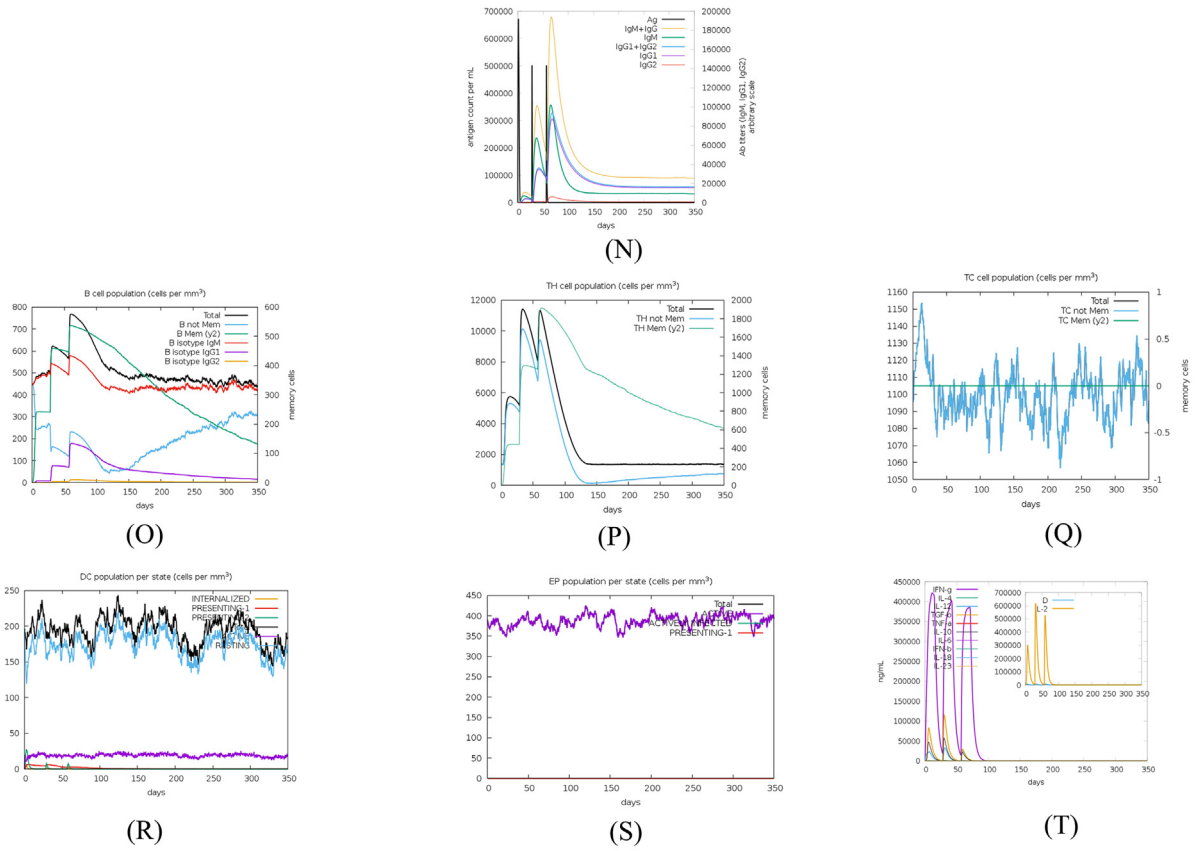

Fig. 8. Showing the IS (immune simulation) results of constructed multi-epitope vaccine. (N) is depicting the antigen antibody relationship as antigen decreased the antibodies level increased also showing the increase in IgG level which is indicator for primary responses. (O) is describing the B-cell population level increasement which indicates secondary and tertiary responses, (P) is depicting the TH-cell population level rise up, (Q) is showing the TC-Cell population level, (R) is showing dendritic-cells which depicts the presence of antigenic peptides on the MHC-I and II, (S) is showing the epithelial cell generation, (T) is describing the interleukins and cytokines concentration level.

reactive immunological response against the SARS-CoV-2 infection. T-cell-produced immunity is long-lasting [78]. MHC-I, MHC-II, and B-cell epitopes were used to create a multi-epitope subunit vaccine. In addition, in the development of the vaccine, the 50S ribosomal protein L7/L12 was employed as an adjuvant, and EAAAK linkers were utilized to connect the L7/L12 (adjuvant) to the B-cell epitope; moreover, GPGPG and AAY linkers were employed to bind the B-cell to the MHC-I and MHC-II epitopes, respectively. Furthermore, there were overlapping sections in B-cell; MHC-I and MHC-II were also merged to eliminate overlap [56]. A total of 7, 10, and 5 MHC-I, MHC-II, and linear B-cell epitopes, respectively, were employed in this multi-epitope construct. The multi-epitope subunit sequence constructed has a molecular weight of 59,974.20 Da based on 563 amino acids. The use of adjuvant linkage with built vaccines improves the construct's immunogenicity [79]. The design was discovered to be antigenic and non-allergenic. Vaccines, like other drugs, have the potential to induce allergic responses. Mild vaccine reactions are frequent, but sometimes they can lead to severe consequences. It has been determined that the most significant barrier to vaccine development is allergenicity [80].

The IEBD Population Coverage tool was used to determine the global coverage of interacting epitopes of MHC-I and MHC-II alleles. There is a divergence in MHC-HLA allele distribution around the globe; therefore, the population coverage of *H. sapiens* MHC-I and MHC-II interacting molecules was conducted, and the following distribution was obtained: 92.06% for Europe, 90.71% for Oceania, 88.28% for North America, 84.66% for East Asia, and 83.77% for Southeast Asia. Our population coverage of 92.06% is in line with the 92.51% coverage reported by Sadat et al. [50]. The vaccination must be 70% effective, and it must be 80% effective to primarily eradicate an epidemic without the need for additional measures [81]. The physio-chemical

characteristics of peptides significantly impact their immunogenicity, transportation, and stability [82]. The current multi-epitope subunit construct has a molecular weight of 59.97420 kDa (59974.20 Da), within the ideal range of 40–70 kDa. The lesser molecular weight of the vaccine is believed to be ideal because the purification process of the vaccine is easier for low molecular weight. The vaccine protein's theoretical pI was discovered to be 8.69, indicating that it is essential. The instability index was determined to be 30.90; a value less than 40 indicates that the structure is more stable [83,84]. The structure's aliphatic index was 89.98, indicating that our construct is thermostable [85]. Its GRAVY was 0.065, indicating a hydrophobic protein [86]. The solubility rate was calculated to be 0.960121, which meant high solubility, an essential indicator that it can be purified easily and produced on a large scale [87]. The secondary structure analysis revealed that 32% of the proteins were α-helix, 11% were beta stands, and 56% were coiled. Furthermore, a total of 62%, 19%, and 18% exposed, medium-exposed, and buried contents, respectively, were discovered.

Disordered domains were identified in 39% of the locations in the existing protein structure. For optimum molecular docking, it is necessary to reduce 3D model inaccuracy and develop a high-quality 3D structure. The 3D structure was refined using GalaxyRefine, and the required features of the structure were developed. Additionally, RAMPAGE findings clearly showed that all parameters were in accordance, suitable for vaccine production. Only those 3D structures with more than 90% of their residues in the favored region are deemed excellent. The protein–protein docking webserver Cluspro2.0 was used to molecularly dock a refined vaccine model with the LBD of the immune receptor TLR-4 (4G8A) since TLR-4 of host immune cells can recognize the viral protein of SARS-CoV-2, which is essential for adaptive immunity [88]. The molecular docking studies revealed stable interactions between the multi-epitope subunit construct and the TLR-4 complex, with a −753.3 kcal/mol; this score is more damaging than the control docking complex energy score of −727.7 kcal/mol reported previously by Safavi et al. [62]. The low energy score of docking is necessary for the efficient binding of vaccines with TLRs [89]. The multi-epitope construct eigenvalue of MDS was 4.816138 e−06, and it climbed steadily in each paradigm during the dynamics (Fig. 6). The variance plot showed that the individual variance decreased in each subsequent mode. These MDS findings exhibit the overall stability of the current vaccine construct + TLR-4 complex [77].

The immunological simulation of a multi-epitope subunit construct demonstrated perfectly normal immune response trends after multiple antigen exposures. The immunoreactivity of the subunit construct was tested by expressing it in the host *E. coli* K12 strain [56]. The server predicted higher B- and T-cell levels after repeated antigen exposure for a longer period. Increased levels of the antiviral cytokines IFN and IL2 indicated the possibility of T-helper cell activation and, consequently, increased Ig production, which supports the humoral immune response [90]. Most importantly, the vaccine design must be expressed in an appropriate *E. coli* strain, to develop recombinant proteins [91,92]. With a CAI of 0.94 and a GC content of 52.39%, the codon optimization method demonstrated high expression in *E. coli* K12. Finally, the multi-epitope subunit vaccine sequence was inserted into the pET-28a vector to efficiently encode the constructed protein in *E. coli* cells. The codon sequence of the multi-epitope subunit construct was inserted between the HindIII (542) and NaeI (1472), which developed a codon of 4843 bp (Fig. 5).

The current study results depicted strong cellular and humoral responses computationally, confirming previous findings that S and N proteins have properties that elicit both cellular and humoral responses. N protein individually generates coronavirus-specific CD8 + T lymphocytes, and SARS-CoV-2 M protein is the most cellularly immunogenic protein [93–97]. Thus, these immunogenic properties of the current structural protein vaccine design suggest that the vaccine may be a better choice for combating SARS-CoV-2 infection. Thus, for the sake of public health, the study's findings should be confirmed as quickly as possible in the laboratory and field.

5. Conclusion

Effective drug development is a time-consuming and expensive procedure; however, the only option for halting the present COVID-19 epidemic is to develop effective and timely vaccines. The use of viroinformatic techniques will undoubtedly aid in developing a rapid and effective SARS-CoV-2 vaccine. This study used various bio- and viroinformatic techniques to design a multi-epitope subunit vaccination. The potentiality of the developed construct was evaluated using immunoinformatics. Thus, an excellent humoral and cellular response was discovered. Furthermore, the engineered subunit construct was successfully colonized in the expression vector pET-28 a (+), demonstrating that vaccine production on a large scale is feasible.

Therefore, the designed construct's true potential has to be validated in the lab and the field on a priority basis in the interest of public health.

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