

Development of a New Epitope Immunogenic Structure Based on the Coronavirus Membrane Glycoprotein M Using Immunoinformatics Tools

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ABSTRACT

Backgrounds: Although conventional therapies have played an essential role in the treatment of many diseases, emerging diseases require new treatment methods with less complications. Therefore, it is important to develop an effective vaccine for infections caused by the coronavirus to prevent mortality and create immunity the community.

Materials & Methods: In this research bioinformatics tools were used to design a vaccine against the M membrane protein of SARS-CoV-2. A total of 27 epitopes confined to B cells and MHC I and II alleles were structurally constructed in M protein for immune stimulation and antibody recognition which were used in the construction of a chimeric peptide vaccine.

Findings: The vaccine was predicted to be a stable, antigenic, and non-allergenic compound. TRL5/vaccine complex analysis and docking simulation indicated a sufficiently stable binding with appropriated receptor activation. The immune response simulation following hypothetical immunization indicated the potential of this vaccine to stimulate the production of active and memory B cells, CD8 + T and, CD4 + T cells, and effective immunological responses induced by Th2 and Th1.

Conclusion: The analysis of in-silico processes showed that the vaccine structure induced high antigenicity and good cellular immunity in the host body and stimulates various immune receptors such as TLR5, MHC I, and MHC II. Vaccine function was also associated with an increase in IgM and IgG antibodies and a set of Th1 and Th2 cytokines. But the final confirmation of the effectiveness of the designed vaccine requires clinical processes.

Keywords: Vaccine, Immunoinformatics, Protein M, Coronavirus.

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Introduction

The unprecedented rapid spread of COVID-19 in December 2019 from Wuhan, China to many countries affected thousands of people, many of whom died within a few months after the initial spread of the disease [1, 2]. SARS-CoV-2 as the causative agent of COVID-19 was identified as a new strain of the genus Beta-coronavirus, with approximately 70% genetic similarity to SARS-CoV [3]. The virus is very similar to the bat corona virus; thus, it is thought to have originated in bats [4, 5]. The viral genome sequence is available in several online biological databases, and this important information for the development of vaccines is available to all researchers. Coronaviruses are involved in causing diseases in mammals and birds. Some of these diseases, such as SARS, MERS, and COVID-19, cause a wide range of respiratory problems, from the common cold and fever to death [6]. Positive single-stranded RNA viruses with a single nucleocapsid are helically symmetric and covered by spike proteins with genome sizes (compared to many coronaviruses) between 27 and 34 kb, which are the largest known viruses among retroviruses [5]. Transmission of the disease along with respiratory problems could lead to death in some people. On the other hand, there is still no effective vaccine to prevent this pathogen in humans [7, 8]. Examination of the structure of the virus has revealed that the M membrane protein plays a key role in virus synthesis by transforming cell membranes into workshops to provide conditions for the formation and production of new viruses. S protein, N protein, and genomic RNA also help regulate the size and diversity of virions by interacting with M protein [10]. Virus particles contain some genetic materials and proteins that affect the structure of the virus to attack host cells. When viral RNA enters the information cell, it is used during the translation process to produce some of

the structural and non-structural proteins required by the virus for replication in the host. The most important structural proteins in these viruses include envelope protein (E), surface glycoprotein (S), membrane protein (M), and nucleocapsid (N). Protein M is the most abundant glycoprotein in virus particles, which is usually considered as an antiviral drug target. There are currently many vaccine candidates for SARS-CoV-2 [9], and the design of epitope-based vaccines may provide a useful complementary approach that directs immunity to immunogenic epitopes in the M protein. Using immunoinformatics, the immunogenic properties of proteins could now be evaluated through high-performance computational methods (INSILICO) [9, 10]. Therefore, a control point and in-silico design methods were used to construct a peptide vaccine against membrane glycoprotein M, as well as to mimic the amplitude of responses in the initial amplification scenario.

Materials and Methods

Preparation of the desired protein sequence from NCBI: Membrane glycoprotein M nucleotide sequences were obtained from the NCBI database via its accession number. **Prediction of linear B-cell epitopes:** FASTA format protein sequencing was required to predict B cell epitopes. Here, three different servers FBCPred, ABCPred, and IEDB were used, and the peptide structures resulting from these servers were considered as final epitopes [11]. **Prediction of MHC-I and MHC-II epitopes and their analysis:** Epitopes of MHC-I and MHC-II receptors were retrieved and selected through ProPred-1, MHC2pred, and IEDB servers and considered as final epitopes [12]. **Design of vaccine structure:** Selected MHC-I, MHC-II, and B-cell epitopes with some linker compounds were used to design the multi-epitope vaccine structure. Adjuvants were added to the end of the vaccine

sequence to increase the vaccine efficacy. Various adjuvants were used to identify human extracellular receptors (TLRs) such as TLR1, TLR2, TLR4, TLR5, and TLR6. One of the adjuvants used was Pam3CSK4 (accession number =2 Z7X_C). The vaccine was then developed by joining adjuvants, MHC-I and MHC-II epitopes, and B-cell epitopes using linker compounds, namely EAAAK, GPG-PG, and AAY. Various studies have shown that administration of adjuvant in vaccine candidate constructs elicits strong T-cell and antibody responses. Therefore, adding adjuvants to vaccines could lead to more effective and longer-lasting immune responses. [13, 14].

Prediction of the vaccine secondary structure: In the process of examining and analyzing the secondary structure of the vaccine construct, Prabi and PsiPred servers were used. At this stage, the secondary structure of the designed vaccine was examined in terms of having alpha-helix, beta-sheet, and random coil structures.

Investigation of allergenic and antigenic properties of the vaccine: VaxiJen v2.0 server and then AntigenPro server were used to evaluate the vaccine antigenicity for validation. Vaccine susceptibility was also assessed through ALLerTPv.2.0 and AllerTOP v.2.0 servers.

Investigation of physicochemical properties and solubility of the vaccine: The physicochemical properties of the candidate vaccine were analyzed via ExPASy-ProtParam server. Also, the solubility of the candidate vaccine was evaluated and predicted through PepCalc and ProteinSol servers, and the surface availability of amino acids was assessed through IEDB server in Kolaskar and Tongaonkar antigenicity section.

Investigation of the third structure of the vaccine and its modification and validation: The tertiary vaccine structure was selected as the superior model among the five models generated by the I-TASSER server

based on the higher negative energy level. Then 3Drefine and GalaxyWEB servers were used to increase the quality of the selected model and modify the structure of the vaccine.

Docking: The docking process was performed to investigate the affinity between the vaccine structure and some human receptors (TLR5, MHC I, and MHC II). The structure of these receptors was retrieved from the RCSB protein database. Then the studied structure was fixed and evaluated by SPDBV, Discovery Studio, and MVD software and used in the docking process. The protein-protein molecular docking process was performed by ClusPro 2.0 server [15]. As a result, several docking complexes with corresponding energy/weight points were obtained, and the best complexes were selected in terms of energy level. The binding process helps select the most suitable vaccine-receptor interaction among several vaccine-receptor complexes. The interactions of the selected final complexes were visualized by PyMOL software, and then the type of links and residues involved in the vaccine-receptor complexes were determined by PDBsum server.

Normal state analysis for structural stability analysis of vaccine-receptor complex: Normal state analysis was performed to evaluate the structural stability of the selected docking complex via the iMODS web server. The server shows NMA mobility with arrows indicating motion direction. Eigenvalues indicate the strength of structures and deformation diagrams of non-reinforced sections in the structure. The server also provides factor B, variance, covariance map, and link matrix of the desired structure.

Immunological simulation: In silico immune simulation was performed using the C-ImmSim server to confirm immunization and immune response induction against the selected vaccine. This server uses a machine learning-based approach to predict epitopes

and related immune interactions. The server automatically simulates three anatomical sections: 1) bone, where stem cells producing blood are stimulated, and myeloid cells are produced; 2) lymphatic organs; and 3) thymus, in which simple T cells are selected to prevent autoimmunity. In this process, three injections containing the designed peptide vaccine were simulated at four-week intervals (i.e., days 0, 28, and 56). Therefore, three injections were performed at intervals of four weeks. However, several injections were performed four weeks apart for repeated exposure to the antigen. In this process, memory T cells as antigen-specific T cells were checked repeatedly, and the indicators obtained from the analysis of this process were analyzed graphically [16].

Codon optimization and preparation and cloning process: Codon optimization was performed to evaluate the expression of the designed vaccine using the codon optimization tool (JCat). Here, *Escherichia coli* (strain K12) was used to optimize the codon. This server provides a codon adaptation index (CAI) that indicates the bias of codon usage and GC content. CAI values between 0.8 and 1 are adaptable, and GC content between 30 to 70% is useful for translation activities.

The pET28a (+) expression vector was also used to simulate in-silico gene sequencing using SnapGene software.

Findings

Results of the desired protein sequence from NCBI: Membrane glycoprotein M nucleotide sequence was obtained from the NCBI database with accession number MT520466.

Prediction of linear B-cell, MHC-I, and MHC-II epitopes and their analysis: At this stage of the work, ProPred-I, MHC2pred, and IEDB servers were used to predict potable epitopes by B and T cells. ProPred-I was used as a basic matrix method for scanning and predicting peptides recognizable by the library of MHC class I alleles, and MHC2pred was used for predicting peptides recognizable by MHC class II alleles, among which the best ones were then selected and prepared for use in vaccine design, the results are presented in the table below, along with their sequences and antigenicity scores (Table 1).

Vaccine design results: At this stage, the chimeric protein of the vaccine was generated from the epitopes screened in the previous steps. To do this, the B cell epitopes

Table 1) Sequence and antigenicity score of the selected epitopes for use in vaccine construct

MHC I Epitopes	Antigenic Score	MHCII Epitopes	Antigenic Score	B Cell Epitopes	Antigenic Score
FLPGVYSV	0.8074	FEKMSVLLSVLLSMQ	0.4583	QYGRSGE	1.3962
YLITPVHV	0.5932	FVMMSAPPAQYELKH	0.9205	ERSEKSYELQ	0.8651
ITSKETLY	0.9644	CLFLLPSLATVAYFN	0.6117	VPGFNEK	1.4732
YLYLTFYL	0.9995	MVSLLSVLLSMQGAV	0.5025	PGFNEKT	1.7546
SLDTYPSL	0.6746	KSAFYILPSIISNEK	0.7169	IKDTEK	1.9949
GLDSLDTY	0.6623	LFFFLYENAFPLPFAM	0.6202	ELDERI	0.6324
FLMSFTVL	0.5616	WSLFFFLYENAFPLP	0.5727	EKTHVQL	1.0571
YLFQHANL	0.5355	QWSLFFFLYENAFPLP	0.4372	THVQLSL	1.6509
LLPSLATV	0.6300	SPFVMSAPPAQYEL	0.5833	HVQLSLPVLQVRDVLVR	0.6734

were first put together and linked by linker structures called EAAAK and KK. Then with the help of GPGPG linker, they were connected to MHC I-based epitopes, which were also connected together with KK linker; finally, a connection was established between the sections containing MHC I and MHC II epitopes using AAY linker. After connecting the structural sequences, an adjuvant compound was added to the end of the generated chimeric sequence (Figure 1).

Results of the vaccine secondary structure prediction: At this stage, the two-dimensional and three-dimensional structure of the produced chimeric composition was examined, and if undesirable folds were created in its structure, the disruptive epitope parts of the chimeric composition were removed from its original structure at the very beginning of the work. With the help of Psipred and Prabi software, the secondary structure of the chimera was investigated. According to the results, the secondary structure of the chimeric composition with 57.40% alpha helix, 36.69% ran-

dom screw, and 5.92% wide string had favorable conditions (Figure 2).

Results of allergenicity and antigenicity: The antigenicity of epitopes selected by IEDB server in Kolaskar and Tongaonkar antigenicity section (Figure 3) was examined. The antigenicity score of epitopes selected for use in vaccine structure was determined by Vaxijen server, it was ensured that all selected epitopes were antigenic in use. Then their allergenicity was evaluated with the help of ALLerTPv.2.0 and AllerTOP software, and those with allergic risk were removed, and those that did not have allergenic properties were isolated and used to make the vaccine structure [17, 18].

Results of physicochemical properties and solubility: The evaluation of the physical and chemical properties of the vaccine was performed by software such as ProtParam, PepCalc, and ProteinSol, which showed an isoelectric pH of 9.94 and a stability of 32.91 for the vaccine construct in the body. It had a suitable half-life of one hour in mammals.



Figure 1) Overview of the structure of the designed vaccine

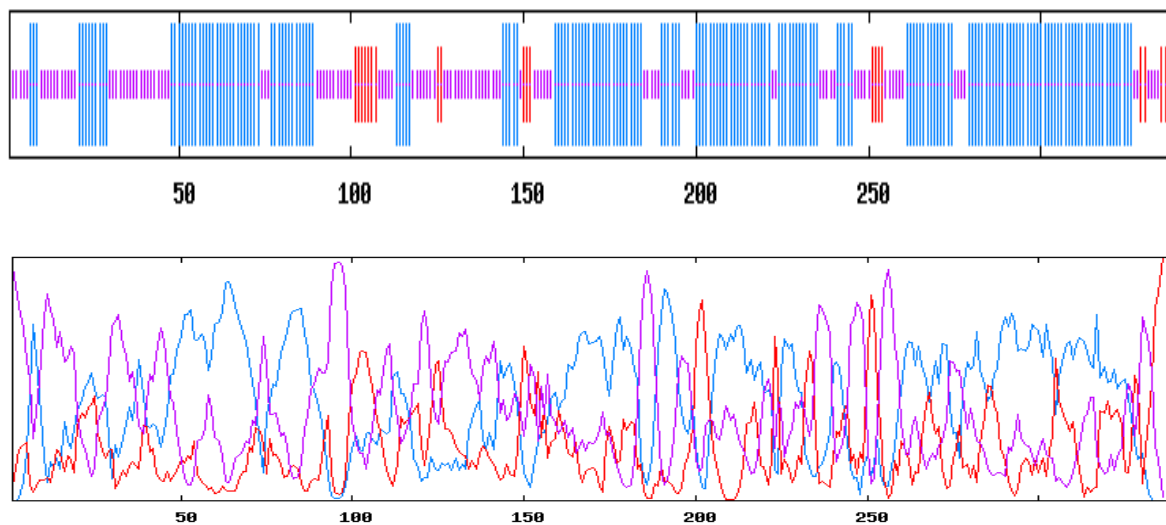


Figure 2) An overview of the condition of the main parts of the secondary structure of the designed vaccine

Also, the vaccine composition had a molecular weight of 39602.20 Da, a gravity score of -0.312, and an aliphatic coefficient of 85.65, it was also in good condition in terms of hydrophobicity and extinction coefficient. According to the results obtained from PepCalc and ProteinSol servers with a score of 0.544, it had good solubility in water, which allows its introduction in laboratory phase processes [19, 20].

Results of the third structure of the vaccine and its modification and validation: To obtain the vaccine three-dimensional structure, modeling methods were used by employing SWISS-MODEL and I-TASSER servers. RMSD was selected as the best model in terms of higher negative energy level among the models provided by the I-TASSER server considering the three desired indicators [21, 22]. The structure of the vaccine on the ProSA server received a score of -2.89, which indicates that the obtained model is in the range of compounds obtained by NMR technology. After modification by the 3Drefine server, the obtained model was placed in a favorable structural position in terms of its amino acids. According to the results of Procheck server and Ramachandran diagram, about 99% of the model structure was in an acceptable and desirable position, which indicates the appropriateness of the obtained vaccine structure model (Figure 4).

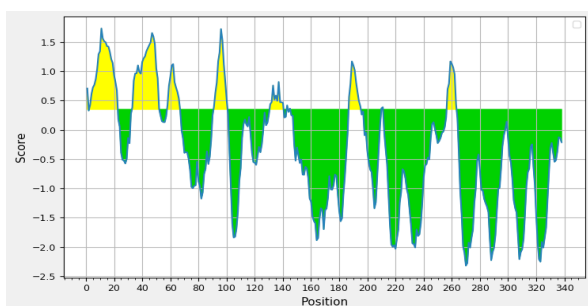


Figure 3) The first 100 nucleotides of the vaccine construct are related to B-cell epitopes. In the diagram above, the antigenicity of the initial sequence of the vaccine structure indicates the high potency of B cell epitopes selected by Kolaskar and Tongaonkar section of IEDB server.

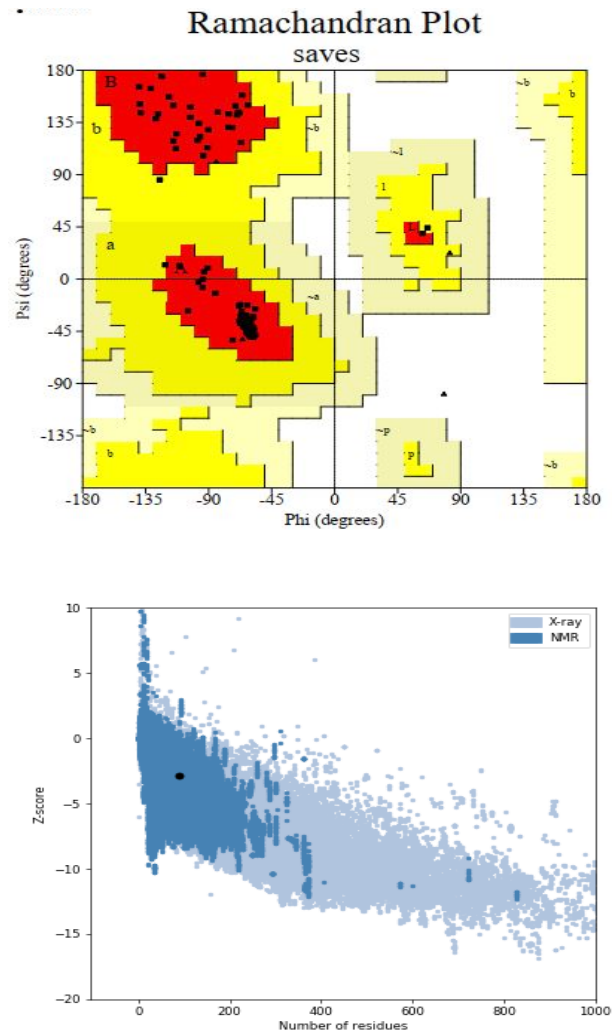


Figure 4) Results of the vaccine model designed by I-TASSER server and validated by ProSA and Procheck servers

Docking results: In this study, receptors used in the docking process, such as TLR4, HLA-A0201, and DRB1.0401, were chosen because of their ability to modulate the immune system, stimulate IFN-g, as well as activate IFN type I responses [23]. Epitopes selected as CD4+T cell epitopes were able to stimulate both Th1 and Th2 cytokines. The docking process was performed between the vaccine construct and TLR4, HLA-A0201, and DRB1.0401 using the ClusPro server. The server created about 25 to 30 possible docking structures with corresponding energy values, from which complexes with the lowest energy scores were selected. The docking between the vaccine

and TLR4 receptor and two human HLA receptors belonging to MHC I and MHC II classes (HLA-A0201 and DRB1.0401, respectively) was analyzed in terms of energy level. TLR4 with an energy level equivalent to 1649.8 kcal/mol established a vaccine. The docking

structures were then visualized using PyMOL software, and the interactions between the designed vaccine and different receptors and residues and the type of links involved in these interactions were analyzed and obtained by the PDBsum server (Figure 5).

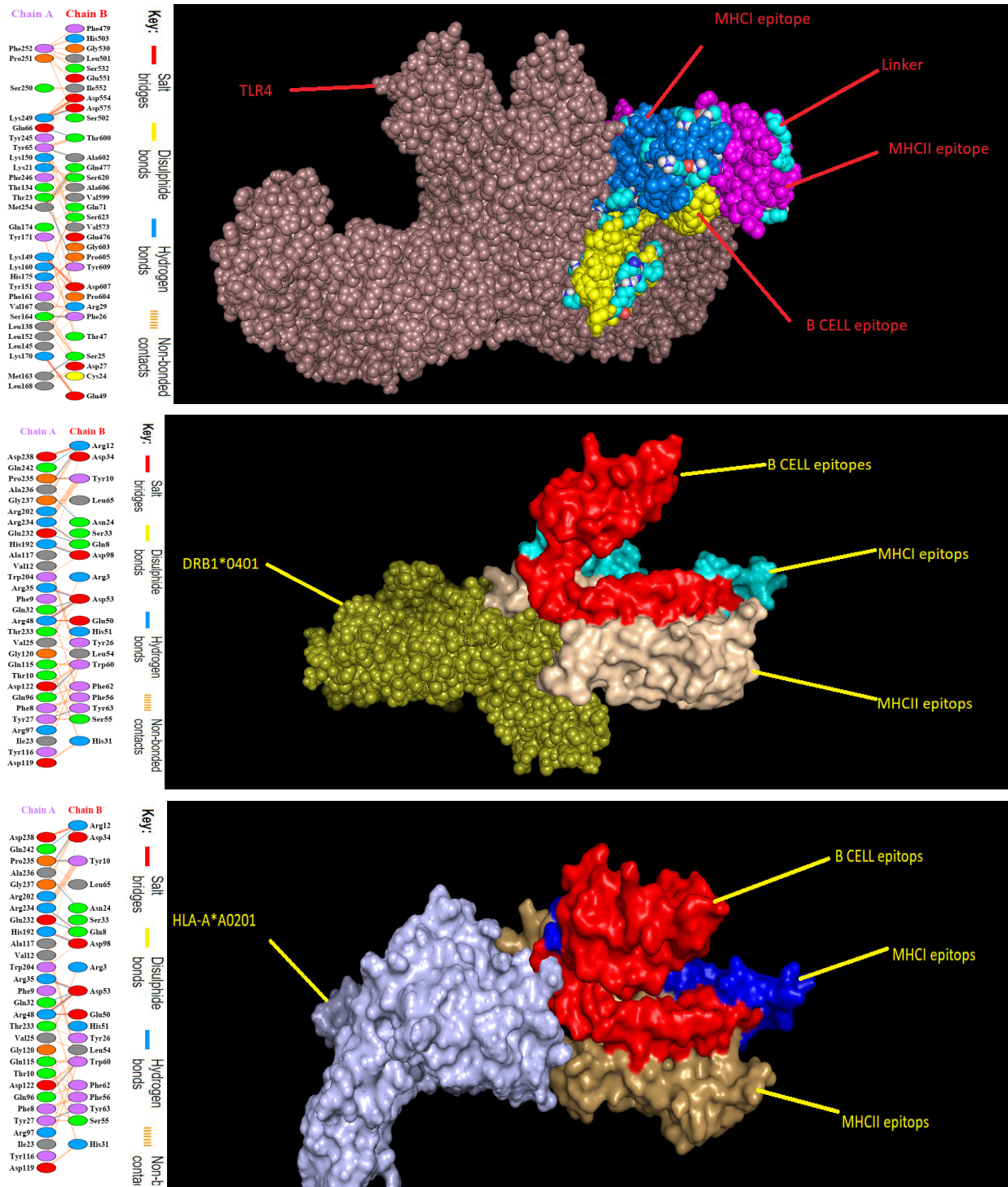


Figure 5: Interactions between the vaccine designed and receptors (TLR4, HLA-A0201, and DRB1.0401)

Results of normal state analysis for structural stability of vaccine-receptor complex: To analyze the biophysical stability and changes of the vaccine-TLR4 complex, molecular dynamics simulations were performed through the iMODs server [24]. The resulting iMOD original chain deformation is shown in Figure 6A. In this figure, the area where the hinges are located has a strong tendency to deform. In Figure 6B, the values of factor B calculated by normal state analysis are proportional to the square root of the mean, and factor B shows the fluctuations of the atomic position and measures the unpredictability of each atom. Figure 6C shows the eigenvalues that are closely correlated with some energies required to flatten the structure, and in general this figure reflects the structural stability of the vaccine-receptor complex, which is shown by eigenvalues.

The eigenvalue of the vaccine-TLR4 complex indicates that less energy is required for deformation of the structure and expression, causing the continuity and stability of the complex to activate immune cascades to remove antigens. The variance was inversely correlated to the eigenvalue. In Figure 6D, the individual variance with red and green represents cumulative variance. The covariance map shows the relationship between the pairs of residues, because the colors red, blue, and white indicate the correlated, anti-correlated, and unrelated pairs of residues (Figure 6E). The elastic lattice diagram shows the pair of atoms attached to the spring (springs), and each point on the diagram represents a connecting spring between a pair of corresponding atoms. In this diagram shown in Figure 6F, the darker gray color indicates the stiffer springs (Figure 6).

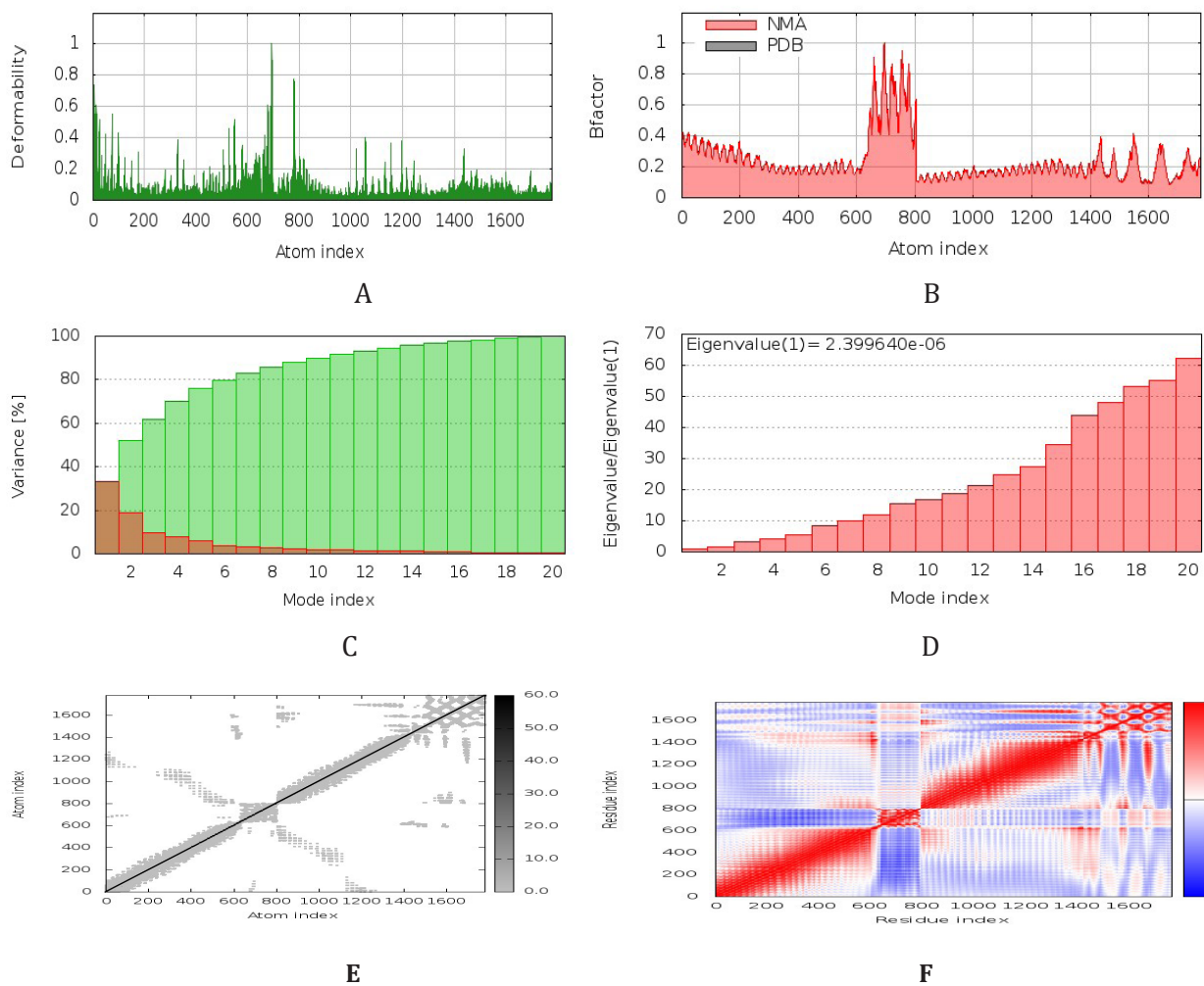


Figure 6) Molecular dynamics simulation of the vaccine-TLR4 complex. The stability of the protein-protein complex was investigated through deformation (A), factor B (B), eigenvalue (C), variance (D), covariance matrix (E), and elastic lattice (F).

Immunological simulation results: The results obtained from the C-ImmSim server were in line with the results of actual immune responses and showed an increase in the production of secondary immune responses. The initial response to the vaccine was demonstrated by increasing IgM levels. In all responses, the increase in B cell population was shown as an increase in IgG1+IgG2, IgM, and IgG+IgM

levels as shown in Figure 7. In addition, the results showed that the process of increasing the proliferation of memory cells occurred in both helper T cells and cytotoxic T cells population (Figure 7D). The results also showed a significant increase in cytokines such as IFN- γ , IL-10, TGF-B, and IL-12. These observations showed that the designed vaccine elicited promising anti-COVID-19 immunogenic reactions (Figure 7).

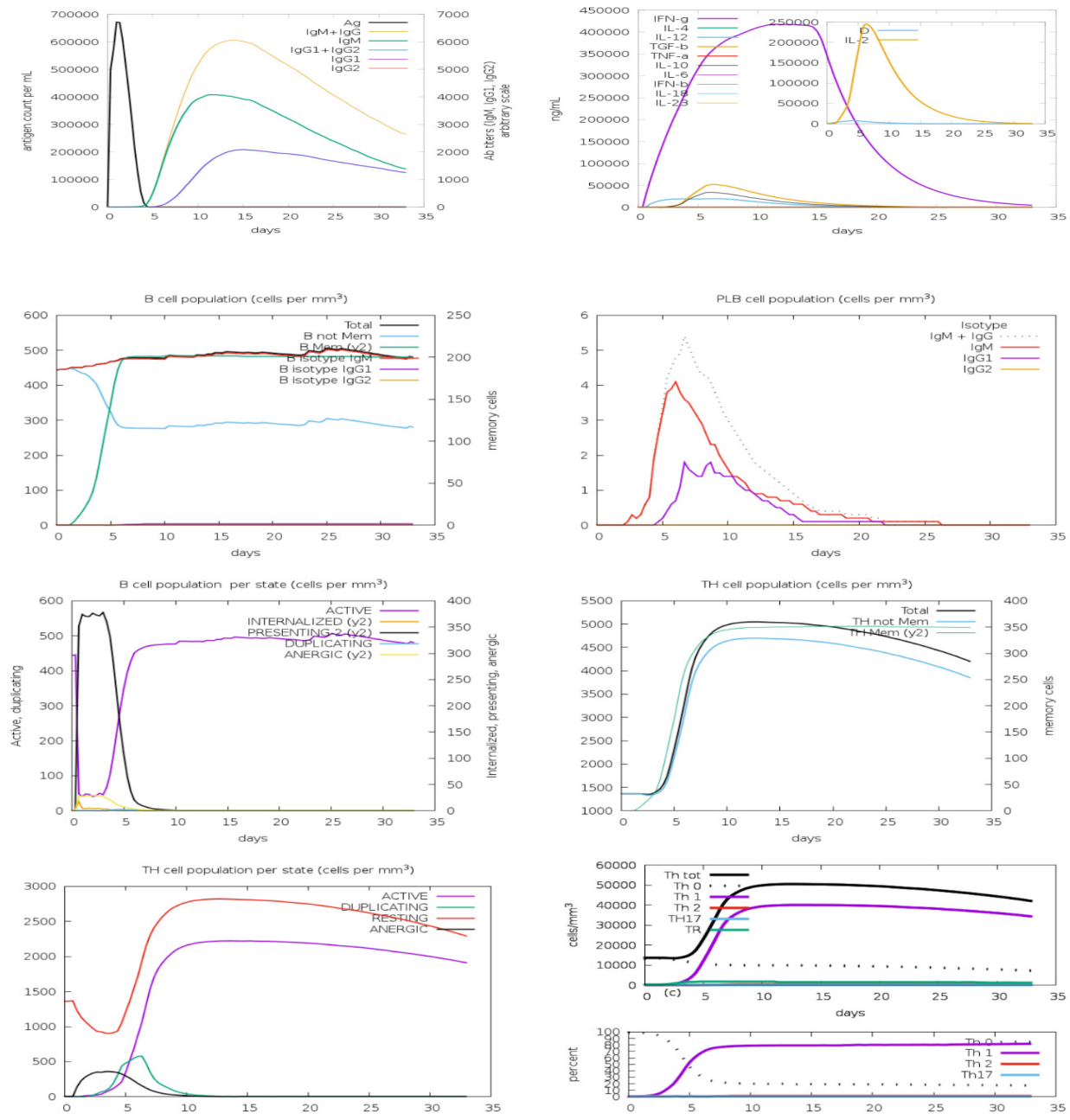


Figure 7) Immunological simulation results provided by C-ImmSim server

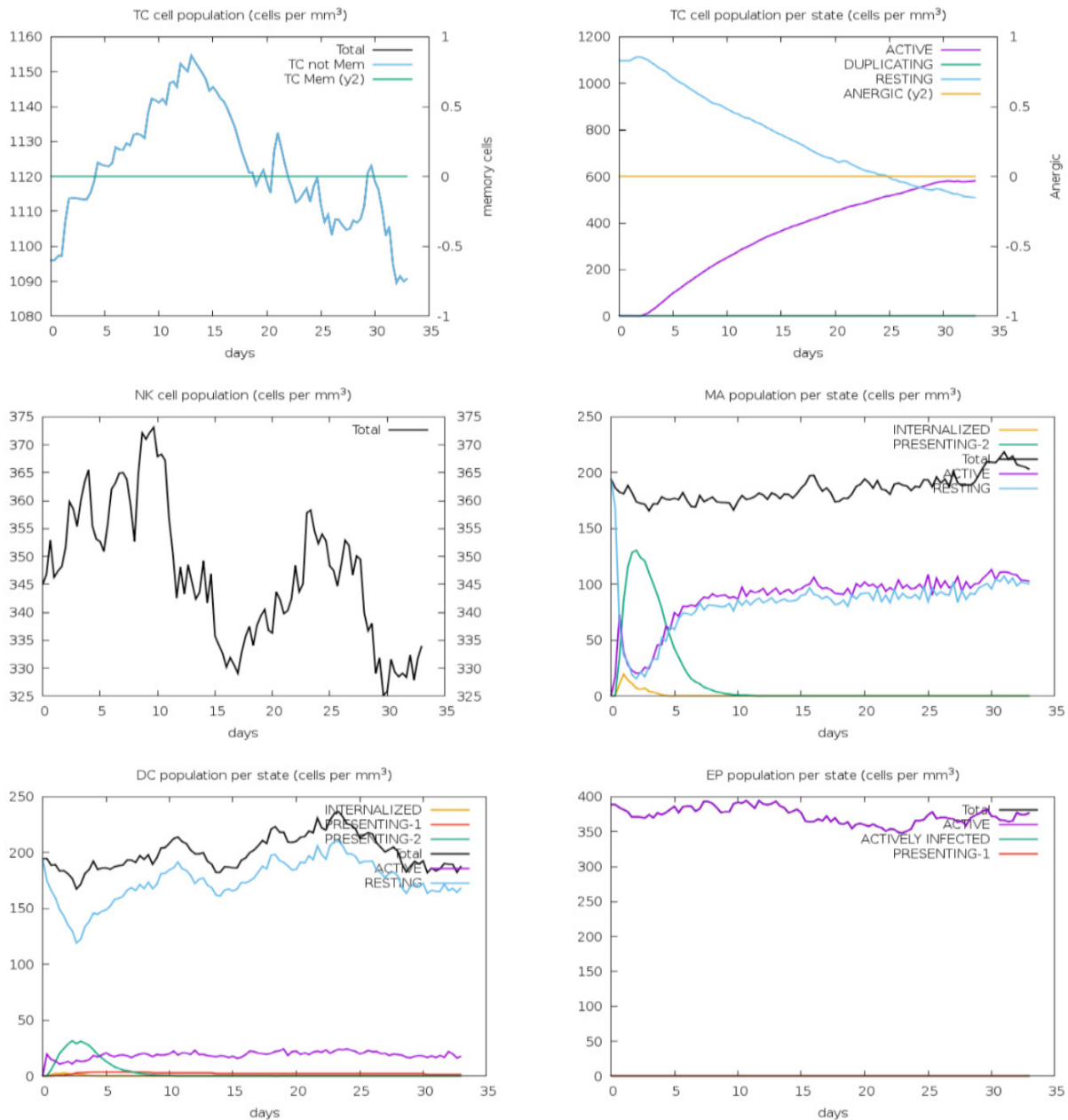


Figure 7) Immunological simulation results provided by C-ImmSim server

Results of codon optimization and vaccine cloning: Vaccine sequences were obtained after codon optimization on the JCat server. The CAI value of the optimized sequences was 1.00, and the GC content was 44.285714285714285%, demonstrating good expression of the vaccine in prokaryotic hosts. Then by inserting the vaccine sequence into the pET28a (+) vector after adding two restriction sites of EcoRI and BamHI restriction enzymes at N and C terminals of

the nucleotide sequence, the vaccine cloning process was carried out using SnapGene software. Also, the accuracy of the cloning process was investigated using SnapGene software based on the gene sequence of the designed vaccine, the vector, and the recombinant structure of the clone resulting from the vector and the gene sequence of the designed vaccine after digestion by two enzymes EcoRI and BamHI in electrophoresis (Figure 8).

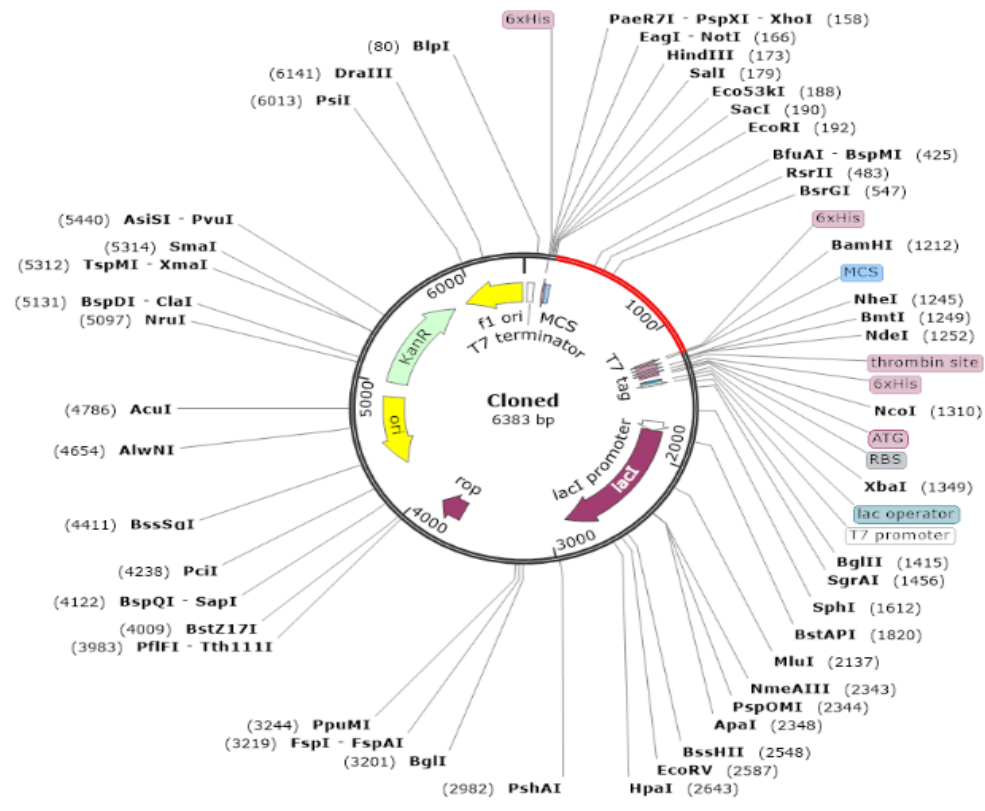
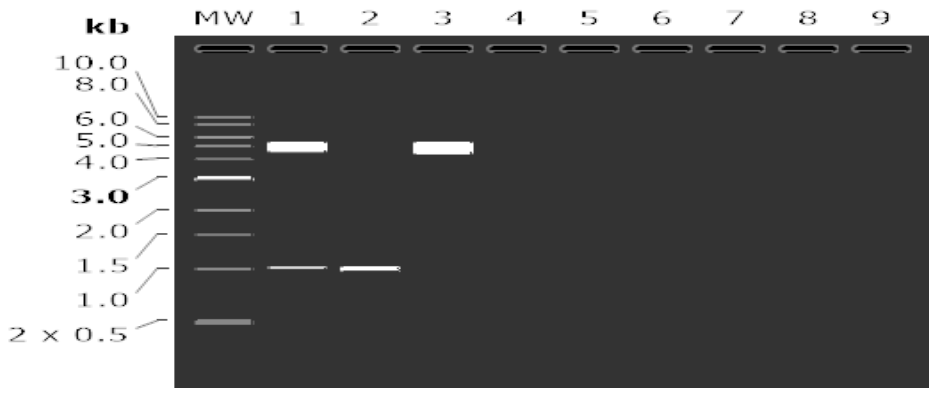
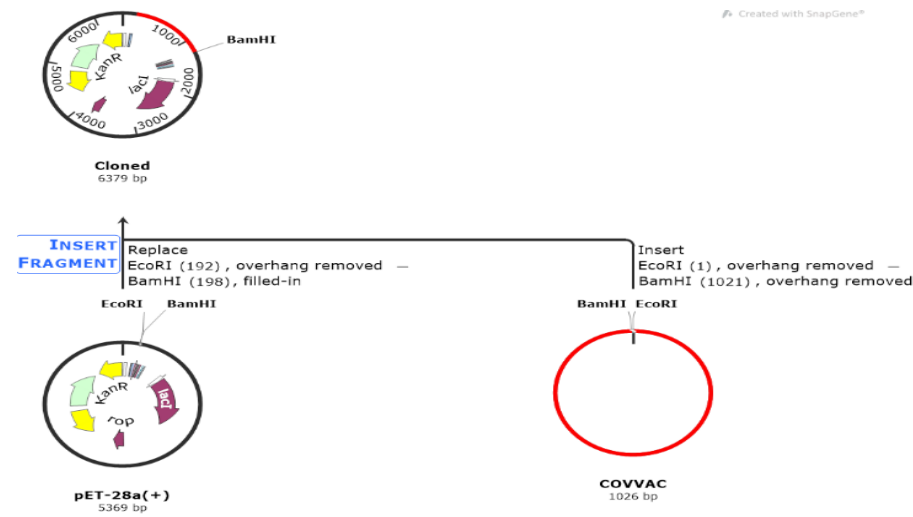


Figure 8) The resulting image and the path of the vaccine cloning and electrophoresis process

Discussion

Today, the epidemic of new viral diseases such as corona is a serious threat that is responsible for the deaths of many people around the world. Thus, there is a serious need for treatment and control measures that could protect people against this type of diseases. Therefore, this study focused on the design of peptide-based vaccines using different epitopes against M membrane protein. We succeeded in creating a peptide vaccine through various steps and in-silico analyses. Epitopes were considered using a set of immunoinformatics tools based on criteria such as stimulation and activation of immune responses without causing toxicity and allergy in the host body. Both groups of B and T cell epitopes were predicted and retrieved in this study to design and construct immunogenic vaccine structures. B cells recognize antigens through antigen receptors, called B-cell receptors (BCRs), which are membrane-bound immunoglobulins [25]. B cell-based epitopes were selected based on surface accessibility through the Kolaskar and Tongaonk antigenicity determination methods via the IEDB server. These peptides were also non-toxic, which makes them a safe vaccine. T cell epitopes that could be recognized and presented by MHC I and MHC II were predicted and selected using different tools. T cell-based epitopes presented by MHC class I molecules are compounds of approximately 8-11 amino acids in length, while MHC class II molecules are capable of delivering longer peptides of 13-17 amino acids in length [26, 6]. CD8 + T cells detect the antigens of a pathogen after binding to MHC I molecules, thus generating a cytotoxic response against the pathogen based on their antigenic properties [27, 12]. Various studies have shown that during this process, specific immunoglobulins are produced against COVID-19, and these compounds have a relatively stable status in patients who have re-

covered from COVID-19 infection [28, 29]. The causative agent of COVID-19, like the causative agents of SARS and MERS, belongs to the genus *Betacoronavirus* of the *Coronaviridae* family. In a study conducted by Ni et al. (2020), it was observed that the immunoglobulins produced against SARS-CoV-2 disappeared in recovered patients after three months [32]. This suggests that immunoglobulins produced against SARS-CoV-2 provide short-term immunity in COVID-19 patients. The results of docking between the immunogenic vaccine construct and MHC II molecules and other immune system receptors demonstrated their ability to stimulate the response of some CD4+ T cell subsets. However, it has been shown that some CD4+ T cell epitopes stimulate subsets of Th cells [26, 30]. Detection of epitopes that are able to induce distinct immune responses through different interactions and residues between the vaccine and receptor is essential in vaccine development. The results of immune simulations showed that both Th1 and Th2 responses could be stimulated by the vaccine through inducing different cytokine responses in vivo. However, it is obvious that the design of this vaccine as a multi-epitope structure has several advantages, and it is possible to achieve both types of immunity in the long run (up to 360 days) if three doses of the vaccine are injected. The results of the docking energy level score between the predicted vaccine construct and MHC II, MHC I, and TLR receptor molecules, which were evaluated comparatively, indicated the formation and establishment of various connections and interactions between them [31, 32]. It was found that the identification of epitopes used in vaccine structures by HLA class II allele molecules is important in eliciting both types of immune responses [23, 26]. This vaccine is predicted to be a stable and soluble compound due to the specific molecular weight, the high pI value of the vaccine

efficacy, as well as the durability of the vaccine structure because proteins with a molecular weight of less than 110 kDa are good candidates for the vaccine [6, 33]. Size and surface properties such as surface charge and hydrophobicity could affect the performance of the designed vaccine candidate because these properties affect the balance between hydrophobicity and hydrophilicity of the vaccine and are therefore very important [34]. The results also showed that the indices obtained from the designed vaccine (such as CAI and GC content) after optimization to achieve high-level protein expression in *E. coli* host had appropriate values for cloning, and these indices mentioned above showed that our designed chimeric structure could be well cloned and expressed in *E. coli*. Finally, based on immunoinformatics evaluations of the designed vaccine, it was determined that this chimeric structure is a safe, soluble, hydrophilic, and stable compound at different temperatures. Therefore, this peptide compound could be considered as a new candidate for inducing immunity against COVID-19 disease. However, the final confirmation of the efficacy of the above vaccine requires *in vitro* and *in vivo* studies.

Conclusion

A new approach to predicting vaccines based on epitopes of coronavirus M membrane protein using bioinformatics tools could target the host's immune system. These antigen-predicting approaches could accelerate the development of a structurally protective vaccine for immunocompromised individuals worldwide. In this research, the designed vaccine was able to stimulate the production of neutralizing antibodies as well as cellular responses after the last dose. The selected epitopes in the form of a vaccine were able to be properly identified by B and T cells and elicit a favorable response against SARC-CoV-2 M membrane protein.

Other compounds that may facilitate the body's immune response to antigens will also be explored in future research.

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Ethical permissions: The study was conducted based on the guidelines approved by the ethics committee, and the ethical rules were followed.

Conflicts of interest: The authors state that they have no conflict of interest with each other, which appears to affect this article.

Authors' contributions: All authors contributed equally in the preparation of different parts of the article.

Fundings: This study was not funded.

Consent to participate: Human patients were not used in this study.

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