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Original Article

A Novel Multi-epitope Vaccine Against SARS-CoV-2 Variants of Concern Strains Applying Immunoinformatics Approaches

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Abstract

Background: The newly emerged coronavirus, SARS-CoV-2, the causative agent of the COVID-19 disease, appeared in Wuhan, China in December 2019 and led to the death of millions of people. The pandemic has increased the demand for effective vaccines and treatments worldwide. Due to the changes in the virus genome caused by mutation, variants with higher transmission ability and pathogenicity have emerged and raised excessive concerns about the efficiency of the developed vaccines. This study aimed to design a multi-epitope vaccine that targets the primary SARS-CoV-2 strain and its five variants of concern using immunoinformatics approaches.

Methods: B-cell, cytotoxic T lymphocytes (CTLs) and helper T lymphocytes epitopes of the conserved and mutated SARS-CoV-2 surface glycoproteins were predicted using immunoinformatics approaches. These epitopes were attached utilizing appropriate linkers, and to provoke the immune response efficiently, the cholera toxin B subunit was added as an adjuvant.

Results: Analyses by different bioinformatics softwares revealed that the designed vaccine's high safety, stability, and efficacy.

Conclusion: In this study, the vaccine designed by immunoinformatics tools showed specific reactivity to Toll-like receptor 4. Given the validation of other epitope-based vaccines, our vaccine might be capable of providing strong immunity in a wide range of populations.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped virus that causes an infectious disease called coronavirus disease 2019 (COVID-19) which was first reported in December 2019 in Wuhan, China and has since spread globally. The Middle East respiratory syndrome coronavirus (MERS-CoV) and the SARS-CoV are two other viruses of this family that were formerly pandemic in 2012 and 2002, respectively.[1-3] As of January 11, 2022, COVID-19 had 313M infected cases, 5.5M deaths, and a devastating global effect on health, economic, and social issues. Therefore, developing effective treatments and efficient vaccines, especially against newly emerged mutated variants is extremely important.[4]

Coronaviruses are categorized into four groups: α, β, γ and δ Coronavirus. The latest Coronavirus is in the beta-coronavirus category.[5,6] The enveloped SARS-CoV-2 has a positive-sense RNA genome encoding 16 nonstructural (NSP1-16) and four structural (Spike, Membrane, Nucleocapsid, and Envelope protein) proteins.[6] Spike protein or surface glycoprotein contains functional S1 and S2 subunits. Subunit S1 contains essential domains that participate in viral tropism by binding to the host angiotensin-converting enzyme 2 (ACE2) receptor. Subunit S2 possesses heptad repeat regions and fusion peptides, which facilitate the integration of the virus with the target cell membranes.[6-8] Since this surface-exposed protein is essential for cell entry, it has been considered a promising candidate for developing vaccines and neutralizing antibodies.[9] On the other hand, due to the lack of efficient treatment for COVID-19, the control of the disease is tied to the development of potent vaccines.

Traditional vaccine development approaches include

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inactivated whole-pathogen and live-attenuated vaccines. Whole-cell vaccines require virus culture and expression of high amounts of proteins and generally need high doses of antigens. Also, conventional methods for developing vaccines require trial and error to examine the immunogenicity of different antigens and their protective efficacy. So, these methods are time-consuming, expensive, and unsuitable for urgent pandemic infections.[10,11] The development of various immunoinformatics tools recently provided a rapid approach for determining immunogenic antigens facilitating the identification of potent vaccine candidates. Multi-epitope vaccines are an emerging approach lacking conventional method limitations.[4,12] In addition, epitope-based vaccines containing small immunogenic portions of the pathogen often induce higher immunity against the infectious agent.[13] These vaccines contain B-cell, cytotoxic T lymphocyte (CTL), and helper T lymphocyte (Th) epitopes, hence having the potential to be considered as a powerful therapeutic tool against pathogens, especially viruses by inducing cellular and humoral immune responses.[14]

As of January 11, 2022, a variety of vaccines have been authorized by the World Health Organization (WHO) for emergency administration, including Pfizer/BioNTech, Serum Institute of India, AstraZeneca AB/SK, Moderna, Janssen, Sinovac, Beijing Institute of Biological Products, Bharat Biotech, and Novavax. While these vaccines are highly effective, the advent of novel SARS-CoV-2 variants has raised excessive concerns about their efficacy. Moreover, their effect on these variants is still unclear.[15]

Based on the WHO announcement, variants of the virus that are associated with one of the following changes have been considered as variants of concern (VOCs): 1- higher transmissibility or harmful transition in COVID-19 epidemiology, 2- enhanced pathogenicity or alteration in the clinical manifestations of the disease, 3- reduction in the efficiency of available diagnostics, vaccines, and therapeutics. These variants include B.1.351 (South Africa), B.1.1.7 (United Kingdom), P.1 (Brazil), B.1.617.2 (Indi), and B.1.1.529 (South Africa), which have multiple mutations in the surface glycoprotein as shown in Table 1.[15-19] Using immunoinformatics methods, this study aimed to develop a multi-epitope vaccine against COVID-19. This innovative vaccine is composed of both conserved and mutant epitopes; hence it could be effective against wild-type and mutant variants of SARS-CoV-2.[20]

Materials and Methods

SARS-CoV-2 sequence and mutation sources

In the current study, the sequences of different variants were retrieved from the NCBI (https://www.ncbi.nlm.nih.gov) database. Sequences include the SARS-CoV-2 Wuhan isolate genome (Accession: NC_045512) along with the five VOC sequences listed as follows: B.1.1.7 (Accession: QUH80290), B.1.351 (Accession: UHN99913), B.1.617 (Accession: UHR12491), B.1.1.529 (ACCESSION: UFO6927) and P.1 (Accession: QUH86629). Also, we utilized the CDC (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html#Concern) and Coronavirus Antiviral & Resistance Database from the Stanford University website (https://covdb.stanford.edu/page/mutation-viewer/#sec_alpha) to retrieve VOCs mutated regions.

Multiple sequence alignment (MSA) and editing

MSA of 500 different SARS-CoV-2 sequences was carried out by NCBI BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) to identify highly conserved regions. BLAST program presents local similar regions by comparing sequence databases to subject protein or nucleotide sequences.

Linear B-cell epitopes prediction

Linear B-cell epitopes were predicted using three servers: (1) BepiPred-2.0 from the IEDB database (tools.iedb.org/bcell/) that utilizes a specific algorithm qualified on epitopes and non-epitopes amino acids to predict B-cell epitopes[21]; (2) BcePred server (webs.iiitd.edu.in/raghava/bcepred/bcepred_instructions.html) which predicts B-cell epitopes using the physico-chemical properties of amino acids, and (3) ABCpred epitope prediction server (webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html) which is constructed upon a recurring neural net.[22]

Prediction of T-cell epitopes

Tepitool (tools.iedb.org/tepitool/) from the IEDB database was applied for CTL and Th epitope prediction.

Table 1. Mutations on the surface glycoproteins of SARSCoV2 VOC strains

Lineages	Location	Characteristic mutations
B.1.1.7 Alpha	United Kingdom (Europe)	Deletion 69–70, deletion 144, Asn501Tyr, Ala570Asp, Asp614Gly, Pro681His, Thr716 Ile, Ser982Ala, Asp1118His
B.1.351 Beta	South Africa	Asp80Ala, Asp215Gly, Lys417Asn, Glu484Lys, Asn501Tyr, Asp614Gly, Ala701Val
P.1 Gamma	Brazil (South America)	Leu18Phe, Thr20Asp, Pro26Ser, Asp138Tyr, Arg190Ser, Lys417Thr, Glu484Lys, Asn501Tyr, Asp614Gly, His655Tyr, Thr1027Ile
B.1.617.2 Delta	India (Asia)	Gly142Asp, Glu154Lys, Lue452Arg, Glu484Gln, Asp614Gly, Pro681Arg
B.1.1.529 Omicron	South Africa	Deletion 69-70, deletion 142-144, deletion 211, insertion 214Glu-Pro-Glu, Ala67Val, Thr95Ile, Tyr145Asp, Leu212Ile, Gly339Asp, Ser371Leu, Ser373Pro, Ser375Phe, Lys417Asn, Asn440Lys, Gly446Ser, Ser477Asn, Thr478Lys, Glu484Ala, Gln493Arg, Gly496Ser, Gln498Arg, Asn501Tyr, Tyr505His, Thr547Lys, Asp614Gly, His655Tyr, Asn679Lys, Pro681His, Asn764Lys, Asp796Tyr, Asn856Lys, Gln954His, Asn969Lys, Leu981Phe

A total of twelve alleles from a variety of human leukocyte antigen (HLA) supertypes including A*02:01, A*03:01, A*01:01, A*26:01, A*24:02, B*07:02, B*27:05, B*08:01, B*39:01, B*15:01, B*40:01, B*58:01 02 were selected for CTL epitopes. Also, the seven-allele method consisting of DRB1*07:01, DRB1*03:0, DRB1*15:01, DRB4*01:01, DRB3*01:01, DRB5*01:01, and DRB3*02:02 alleles was used for Th epitopes. The length of epitopes for MHCI was 9 amino acids and for MHCII was 15 amino acids. TepiTool performed binding prediction of peptides to MHC class I and II in six steps.[23]

The design and construction process for the final vaccine

After determination of T-cell and B-cell epitopes, they were attached with KK and GPGPG linkers, respectively. Furthermore, an EAAAK linker was used to attach the cholera toxin B (CTB) subunit to the N-terminal of the vaccine. In order to determine similarity with Homo sapiens (taxid:9606) proteins, the final vaccine sequence were analyzed with BLASTp on the NCBI database (blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE = Proteins).

Evaluation of worldwide population coverage

The population coverage was estimated via the Epitope Analysis Tools section of the IEDB database (tools.iedb.org/population/). This tool is based on the frequencies of the HLA genotype and MHC binding data.[24]

Assessment of antigenicity, toxicity, and allergenicity

The antigenicity of each epitope and the final vaccine was estimated by Vaxijen v2.0 (ddg-pharmfac.net/).[25,26] ToxinPred was utilized to check the toxicity of each epitope and the final vaccine. (webs.iiitd.edu.in/raghava/toxinpred/index.html). ToxinPred is an immunoinformatic server for prediction of peptide and protein toxicity. To assess the allergenicity of the final vaccine and its epitopes, AllergenFP 1.0 (ddg-pharmfac.net/AllergenFP/) and AllerTOP v. 2.0 (ddg-pharmfac.net/AllerTOP/index.html) were used.[27]

Physicochemical properties analysis and solubility prediction

ExPASy ProtParam server (web.expasy.org/protparam/) was employed for physico-chemical properties prediction of the vaccine and each selected peptides. ProtParam is a computational tool to determine diverse physico-chemical properties of a protein sequence. Solubility was assessed by SOLpro (scratch.proteomics.ics.uci.edu/). SOLpro server is a tool for predicting protein solubility upon overexpression of proteins in *Escherichia coli*.[28]

Prediction of solvent accessibility and secondary structure

The final vaccine secondary structure was evaluated by PSIPRED (bioinf.cs.ucl.ac.uk/psipred/). PSIPRED is a method to predict the precise secondary structure of proteins.[29] It has three secondary structures: α-helix (H), β-sheet (E) and coiled regions (C). These three structures combine to form the tertiary structure of the vaccine, and determine the overall shape of the vaccine. This shape is important for the function of the vaccine, as it determines how the vaccine interacts with other molecules. In addition, RaptorX (raptorx.uchicago.edu/StructurePropertyPred/predict/) predicts solvent accessibility (ACC) and disorder regions (DISO). ACC determination indicates the amount of protein exposed to water molecules. Furthermore, a DISO evaluation can be used to determine the flexibility and disorder of the protein. The RaptorX Property online server without utilizing any template information can predict the structural properties of a protein sequence.[30]

Three-dimensional (3D) modeling and evaluation

The RaptorX server was utilized for the final vaccine 3D structure prediction (raptorx.uchicago.edu/ContactMap/). RaptorX predicts protein interactions and structures.[31] In order to refine the selected model, GalaxyRefine (galaxy.seoklab.org/cgi-bin/submit.cgi?type = REFINE) was utilized. This server is a tool for enhancing local and overall structure qualities.[32] The final refined structure was validated by ProSA (prosa.services.came.sbg.ac.at/prosa.php). ProSA is a helpful tool for a wide range of protein 3D structures to find possible errors.[33]

Prediction of conformational B-cell epitopes

For prediction of conformational B-cell Epitopes, we used the ElliPro: Antibody Epitope Prediction tool from the IEDB database (tools.iedb.org/ellipro/). This server uses protein structures or sequences to identify discontinuous antibody epitopes.[34]

Analysis of the binding mode and affinity of the final vaccine

Docking analyses were conducted using the ClusPro 2.0 server (cluspro.bu.edu/login.php). ClusPro 2.0 is an online server that uses a combination of shape-based and energy-based algorithms to predict the binding affinity of a protein-ligand complex. ClusPro 2.0 analyzes the 3D structure of the proteins, predicts the binding sites and the potential interactions between the proteins and the ligands.[35] Docking was conducted between an immune receptor (Toll-like receptor4) and the refined vaccine.

Virtual cloning in Escherichia coli

As a part of the cloning of the vaccine fragment, the Java Codon Adaptation Tool (jCat) (jcat.de/) was utilized to optimize the codons of selected vaccine.[36] Finally, using Snap Gene software version 3.2.1 RRID: SCR_015052, the optimized sequence was inserted into the PET28a + vector.

Results

Sequence and mutant variants sources

The primary SARS-CoV-2 (Wuhan isolate) and VOCs protein sequences were acquired from the NCBI

database: primary strain (Accession: YP_0097243900), B.1.351 (Accession: UHN99913), B.1.1.7 (Accession: QUH80290), B.1.617 (Accession: UHR12491), P.1 (Accession: QUH86629) and B.1.1.529 (Accession: UFO6927). Also, VOC mutations were retrieved from the CDC and Stanford University databases. VOC mutations are located in structural and non-structural proteins. Due to the critical function of surface glycoprotein in cellular entry, this protein is considered as our primary target antigen for analysis (Table 1).

Multiple sequence alignment and editing

The NCBI alignment tool was used for sequence alignment of 500 spike glycoprotein sequences to distinguish highly conserved and mutant regions (Supplementary File 1).

Linear B-cell epitopes prediction

B-cell epitopes are peptides that react with B-cell progenitors and stimulate humoral immune responses.[37] Several B-cell epitopes against conserved and mutant regions of spike protein were identified by three B-cell epitope prediction servers, BepiPred-2.0, BcePred, and ABC pred. BepiPred-2.0 was considered as the main server, and epitopes that are not identified by all three servers were discarded. Highly conserved epitopes and epitopes with the mutation were selected; among the VOC, only B.1.1.7, B.1.617, and B.1.1.529 variants have epitopes in mutant regions. Eventually, those epitopes with a high score and high antigenicity were selected as the final epitopes (Table 2).

Prediction of T-cell epitopes

CTLs are one of the most vital components of the adaptive immune system. Specific peptides from foreign sources bind to MHC molecules, and then T-cell receptors recognize the MHC-peptide complex to provoke an immune response.[38] As a result of the MHC class I signal, CTL is able to form complexes with peptides presented by MHC.[39] MHC-I epitope prediction was accomplished for cytotoxic T-cells by TepiTool from the IEDB server for highly conserved and mutant regions. Twelve alleles from different HLA supertypes including A*02:01, A*01:01, A*03:01, A*24:02, A*26:01, B*07:02, B*27:05, B*08:01, B*40:01, B*39:01, B*15:01, B*58:01, were chosen. Among the predicted epitopes, those that reacted with multiple HLA and had high antigenicity were selected. All VOC except B.1.617 had MHCI epitopes in mutant regions (Table 3).

Helper T lymphocytes (Th) recognize MHC class II surface peptides. The 7-allele method including DRB1*07:01, DRB1*03:0, DRB1*15:01, DRB4*01:01, DRB3*01:01, DRB5*01:01 and DRB3*02:02, was chosen for MHC class II epitope prediction. The predicted conserved and mutant Th epitopes was shown in Table 4. Among VOC only the B.1.1.529 variant had MHCII epitopes.

Construction of the final vaccine

After analysis of epitopes, 9 B-cell, 39 CTL, and 6 Th epitopes were confirmed to be included in the final vaccine. B-cell and T-cell epitopes were attached with GPGPG and KK linkers, respectively. A suitable adjuvant will increase the immune response as well as reduce the amount of antigen and vaccine dosage. To achieve these benefits, the cholera toxin B subunit (CTB) protein was considered as an adjuvant. CTB is known to trigger B and T-cell immune responses.[40] The final vaccine contained 532 amino acids in length. Based on BlastP results between the final vaccine sequence and the Homo sapiens (taxid:9606) proteome, no significant similarity was found, which suggests that the vaccine is not likely to cause autoimmunity.

Evaluation of world population coverage

HLA or MHC molecules are significantly polymorphic. Up to now, a large diversity of HLA alleles has been described. In diverse ethnicities, the HLA alleles expression occurs at drastically different frequencies. Therefore, in the design

Table 2. Final B-cell Epitopes

Epitopes	Position	Allergenicity	Antigenicity	Toxicity
Conserved				
GVLTESNKKFLPF	550-562	NON-ALLERGEN	0.9976 Probable antigen	Non-toxin
GVSVITPGTNTSNQVA	594-609	N-A	0.4651 Probable antigen	N-T
ECVLGQSKR	1031-1039	N-A	0.9377 Probable antigen	N-T
B.1.1.7				
NVTWFHAISGTNGTKRFDNPVLP	61-83	N-A	0.6026 Probable antigen	N-T
B.1.617				
NYNYRYRLFRKSNLKP	448-464	N-A	0.6178 Probable antigen	N-T
B.1.1.529				
YFASIEK	89-95	N-A	0.4914 Probable antigen	N-T
DPFLDHKNNKSWMESE	136-151	N-A	0.6031 Probable antigen	N-T
AWNSNKLDSKVS	432-443	N-A	0.6750 Probable antigen	N-T
SYQTQTKSHRRARSVA	670-685	N-A	0.8528 Probable antigen	N-T

Table 3. Conserved and mutated MHC class I epitopes

Epitopes	Position	Percentile rank	HLA Allele	Allergenicity	Antigenicity	Toxicity
Highly conserved						
QYIKWPWYI	1208-1216	0.01 0.58	A*24:02 B*58:01	Non-allergen	1.4177 Probable antigen	Non-toxin
VYDPLQPEL	1137-1145	0.04 0.14 0.16 0.77 0.8 0.99	A*24:02 B*39:01 B*08:01 B*07:02 A*02:01 B*58:01	N-A	0.4525 Probable antigen	N-T
ILDITPCSF	584-592	0.5 0.76 0.83 0.89	A*01:01 B*15:01 A*24:02 B*08:01	N-A	1.1835 Probable antigen	N-T
GVVFLHVTYV	1059-1068	0.11 0.22 0.31	A*02:01 B*15:01 A*26:01	N-A	1.4551 Probable antigen	N-T
QIITTDNTF	1113-1121	0.26 0.44	B*15:01 A*26:01	N-A	0.4253 Probable antigen	N-T
LLFNKVTLADAGFIKQY	821-837	0.08 0.28 0.57	A*02:01 A*03:01 A*26:01	N-A	0.4165 Probable antigen	N-T
Epitopes common with highly conserved B-cells						
ESNKKFLPF	554-562	0.2	A*26:01 B*08:01	N-A	1.0278 Probable antigen	N-T
GVLTESNKK	550-558	0.4	A*03:01	N-A	0.8797 Probable antigen	N-T
B.1.1.7						
CNDPFLGVY	134-142	0.58	A*01:01	N-A	0.4295 Probable antigen	N-T
NSHRRARSV	676-684	0.8	B*08:01	N-A	0.6231 Probable antigen	N-T
B.1.351						
APGQTGNIADYNYKL	411-425	0.18 0.24 0.52	B*15:01 A*02:01 B*07:02	N-A	1.2926 Probable antigen	N-T
P1						
GAEYVNNSY	652-660	0.11	A*01:01	N-A	0.7663 Probable antigen	N-T
B.1.1.529						
GVYFASIEK	87-95	0.02	A*03:01	N-A	0.4008 Probable antigen	N-T
RSYSFRPTY	490-498	0.06 0.08 0.18 0.42 0.52	B*15:01 B*58:01 A*03:01 A*01:01 A*26:01	N-A	0.9553 Probable antigen	N-T
YNLAPFFTF	366-374	0.29 0.75	A*24:02 B*58:01	N-A	0.9319 Probable antigen	N-T
DLPQGFSAL	212-220	0.41 0.56	B*08:01 A*26:01	N-A	0.5622 Probable antigen	N-T

of multi-epitope vaccines, choosing epitopes with various HLA binding particularities will provide expanded population coverage. Hence, the determination of proper groups of HLA alleles for this purpose to achieve high population coverage is extremely important.[24] To consider this vital issue, the population coverage of our final vaccine was assessed using the IEDB database for class I and class II MHC alleles (Figure 1). The coverage prediction tool indicated that the final vaccine has 93.03% world coverage, and the highest coverage belongs to Sweden, Ireland South, Ireland Northern, England, Finland, and Germany respectively (Table 5). Also, in some regions such as North America, Asia, South America, and Europe, with the most elevated number of patients and deaths, the designed vaccine showed a high coverage (Table 6).

Assessment of antigenicity, toxicity, and allergenicity

Determination of epitopes which are crucial for inducing an immune response is an essential step in multi-epitope vaccine development.[26] For this purpose, the antigenicity prediction of each epitope and final vaccine sequence was performed via the Vaxijen 2.0 server, and epitopes that were predicted as non-antigen were eliminated. The final vaccine antigenicity score was 0.5484, which

Table 4. Conserved and Mutated MHC class II Epitopes

Peptide start-end	Peptide	Median consensus percentile	Allergenicity	Antigenicity	Toxicity
Highly conserved					
1216-1230	IWLGFIAGLIAIVMV	18.0	Non-allergen	0.6150 Probable antigen	Non-toxin
1061-1075	VFLHVTYVPAQEKNF	20.0	N-A	1.0339 Probable antigen	N-T
1016-1030	AEIRASANLAATKMS	13.0	N-A	0.8255 Probable antigen	N-T
Epitope common with highly conserved MHCI epitope					
821-835	LLFNKVTLADAGFIK	19.0	N-A	0.6327 Probable antigen	N-T
B.1.1.529					
481-495	AGFNCYFPLRSYSFR	15.0	N-A	1.0073 Probable antigen	N-T
361-375	DYSVLYNLAPFFTFK	13.0	N-A	0.7838 Probable antigen	N-T

Table 5. Populations with the highest coverage for the selected HLA alleles

Population/area	Coverage (%)
Sweden	99.15
Ireland South	99.32
Ireland Northern	99.08
England	99.0
Finland	98.65
Germany	98.69
World	93.03

Table 6. Populations with the most elevated number of patients and deaths caused by COVID-19

Population/area	Cases	Deaths	Region	Coverage (%)
United States	63,390,876	863,896	North America	93.45
India	36,070,510	484,655	Asia	80.01
Brazil	22,630,142	620,281	South America	81.06
United Kingdom	14,732,594	150,609	Europe	97.67 (Europe)
France	12,573,263	126,059	Europe	97.02
World	314,202,049	5,521,760	-	93.03%

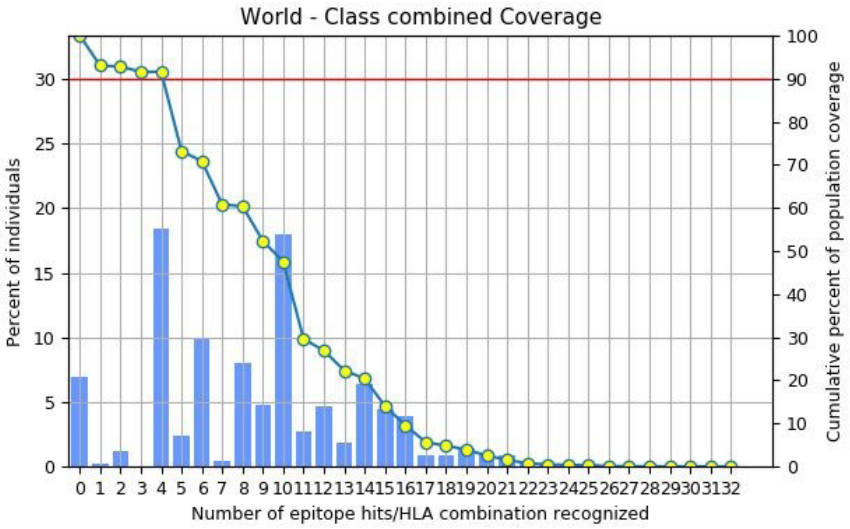

Figure 1. Population coverage of MHCI and MHCII epitopes

confirms its high antigenicity. The predicted results are shown in Tables 3 and 4. Toxicity and allergenicity are other important considerations in vaccine development. The ToxinPred, AllergenFP 1.0, and AllerTOP servers revealed that the final vaccine and its epitopes are non-toxic and non-allergenic.

Physicochemical properties analysis and solubility prediction

The physico-chemical properties and predicted factors are summarized in Table 7. The final vaccine had a molecular weight of 59129.31 Da, an isoelectric point of 9.95, and an estimated half-life of more than 30 hours in mammals. The instability index (ii) value lower than 40 describes stable proteins. Vaccine instability index was predicted to be 31.82 and classified as stable. The globular proteins in the final vaccine were more heat-stable, as indicated by the aliphatic index of 69.87. It was determined that the final vaccine was hydrophilic with a Grand average of hydropathicity (GRAVY) of -0.468, which suggests a strong interaction between the vaccine and water molecules.[41] As predicted, the final vaccine has a solubility of 0.706369, which indicates a proper value. All parameters are shown in Table 7.

Prediction of Solvent Accessibility and Secondary

Table 7. Physicochemical propriety of the final vaccine

Molecular weight	Theoretical isoelectric point (pi)	Aliphatic index	Instability index	Solubility	Grand average of hydropathicity (GRAVY)	Half-life
59129.31	9.95	69.87	31.82 stable	0.706369	-0.468	More than 30 hours in mammalian reticulocytes (in vitro), more than 10 hours in Escherichia coli (in vivo), and more than 20 hours in yeast (in vivo)

Structure

The secondary structure as shown in Figure 2 was predicted by PSIPRED. Results indicated that the final vaccine had 29% helix, 53 % coil, and 18% strand. RaptorX property server was used to predict the ACC and DISO regions. Results indicate that 52% of the vaccine were exposed, 20% were medium exposed, 27% were buried, and only 10% of positions were identified as disordered (Figure 3).

Three-dimensional modeling and evaluations

Based on the RaptorX server, initial 3D structural modeling of the final vaccine with 532 amino acids was performed. GalaxyRefine was then utilized to refine the selected model (Figure 4). According to root mean square deviation (RMSD), global distance test-high accuracy (GDT-HA), MolProbity, poor rotamers, Rama favored and Clash score, Model 1 was selected as the final refined vaccine. GDT-HA high score indicates high similarity between refined models and initial structure so Model 1 with GDT-HA score value of 0.9431 was selected as the final refined 3D structure. A lower RMSD score demonstrates higher stability and the selected model show score of 0.434. Clash score, MolProbity, and Rama favored indicate, the unfit all-atom steric overlappings, crystallographic resolution, and the size of energetically favored regions of the models, respectively. Lower clash score and MolProbity and Rama favored greater than 85% are appropriate. Model 1 with a Clash score value of 15.9, MolProbity value of 2.326, and Rama favored value of 87.0 was an acceptable choice (Table 8). Finally, the selected refined 3D model was validated using the ProSA tool to find potential errors. ProSA calculated a Z-score of refined vaccine value of -6.94, which indicates that the final vaccine structure is within an acceptable range generally located for native proteins of related size, demonstrating an appropriate overall model quality (Figure 5a). The residue score and the local vaccine model quality are also predicted using ProSA as shown in Figure 5b. Generally, positive values correlate with inappropriate or inaccurate regions of the input structure.[42]

Prediction of conformational B-cell epitopes

Based on the refined 3D model, the ElliPro server predicted six conformational B-cell epitopes with scores ranged from 0.789 to 0.532. All epitopes structures and related detailed information were shown in Figure 6 and Table 9.

Analysis of the binding mode and affinity of the final vaccine

The docking analysis was performed using ClusPro v2.0. in order to study the binding mode and stability of the final refined vaccine structure. In response to pathogen-associated molecular patterns, toll-like receptors (TLRs) trigger immune responses. Therefore, TLR4 (PDB ID: 4G8A) was selected as a docking receptor for molecular docking.[43] Among the displayed models, a model with the lowest energy score of -1363.5 which indicates appropriate binding affinity of the receptor and ligand was selected as the final docked complex (Figure 7).

Virtual cloning in Escherichia coli

The final sequence of the vaccine was reverse transcribed and codon-optimized using JCat before being expressed in Escherichia coli (strain K12). Based on a GC-content of 48.3% and a Codon Adaptation Index Value of 1.0, the optimized 1596 bp vaccine sequence demonstrated high expression efficiency in the *E. coli* host. After that, the optimized fragment was inserted into the PET28a + expression vector using Snap Gene software. The construct had a total length of 6929 bp, including the inserted vaccine sequence (red in Figure 8) between restriction enzymes (BamHI and XhoI) cleavage sites. The details of cloning have shown in Figure 8.

Discussion

Considering the devastating effects of the current COVID-19 pandemic on public health, vaccine development has become an urgent need for control of the pandemic. Various types of vaccines are currently authorized for emergency administration. However, there are serious concerns about their efficiency against the emerging VOCs strains containing different surface glycoprotein mutations.[15] Recent studies have also revealed that neutralized antibody titers are reduced for the emerging VOCs.[44,45] Traditional and modern approaches have been adopted for COVID-19 vaccine development. While traditional methods such as inactivated whole-pathogen and live-attenuated vaccines, have been approved and are widely used, most COVID-19 vaccines are based on modern approaches (subunit vaccines, DNA vaccines, mRNA-based vaccines, and viral vector-based vaccines). Although subunit and multi-epitope vaccines have their limitations, such as the lack of bioinformatics tools for prediction of specific antigen processing sites and the need for animal studies to confirm the vaccine efficacy, these vaccines are more precise in targeting the virus, since they contain only the portion of the virus required to elicit an efficient immune response. Additionally, they

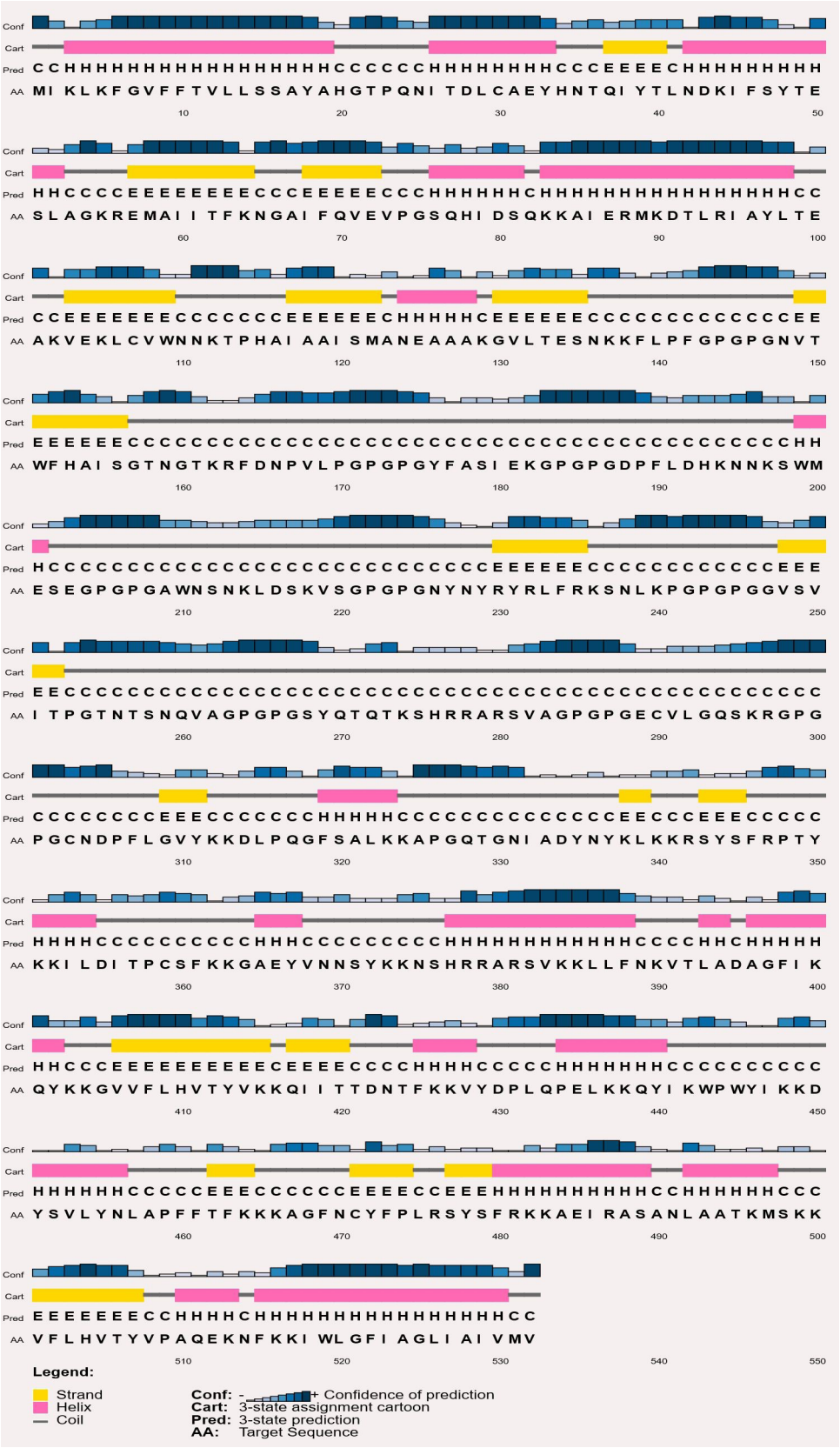

Figure 2. Vaccine secondary structure

are more suitable for large scale production without direct exposure to live viruses.[46,47] These findings prompted us to develop an immunoinformatics-based multi-epitope vaccine against SARS-CoV2 and emerging VOC strains. For this purpose, we constructed a non-allergic and non-toxic multi-epitope vaccine containing T-cell and B-cell specific immunogenic epitopes of SARS-CoV-2 (Wuhan strain) and its VOC strains. According to

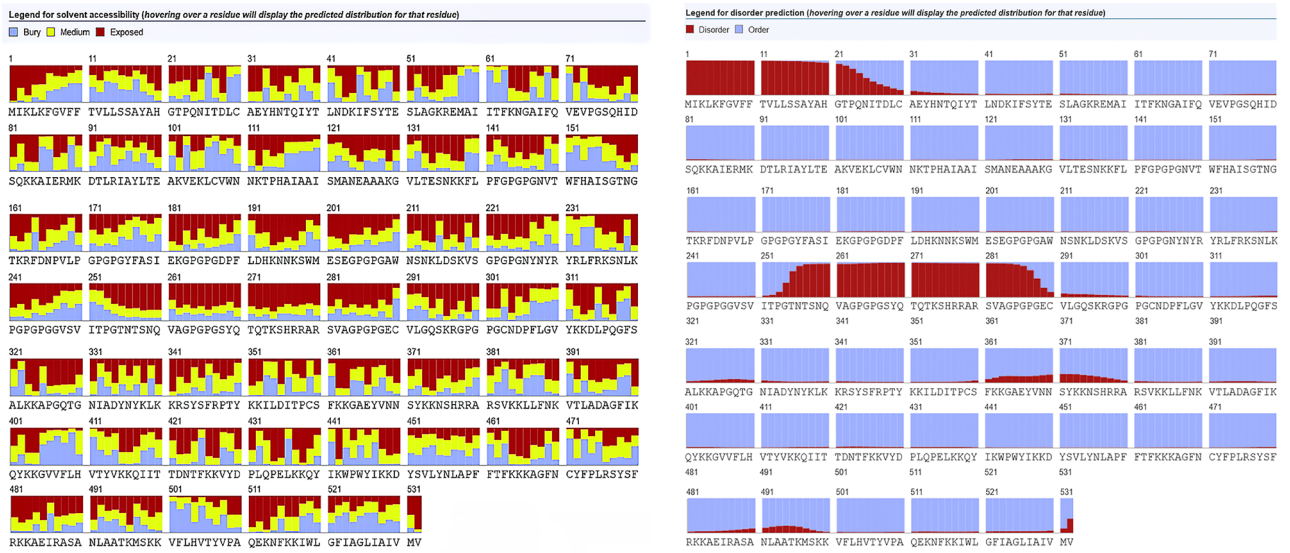

Figure 3. Vaccine solvent accessibility (ACC) status and disorder regions (DISO)

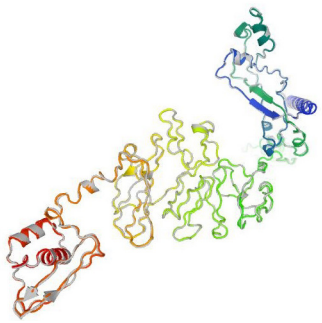

Figure 4. 3D modeling structure of designed vaccine

Table 8. Structural information of refined vaccine models

Model	RMSD	GDT-HA	MolProbity	Rama favored	Clash score
Initial	0.000	1.0000	2.763	86.6	35.2
Model 1	0.434	0.9431	2.326	88.5	15.9
Model 2	0.426	0.9474	2.351	89.7	17.7
Model 3	0.431	0.9422	2.322	88.3	16.3
Model 4	0.446	0.9380	2.315	89.7	15.8
Model 5	0.443	0.9356	2.306	88.9	15.0

different methods of analysis, the aimed vaccine is highly immunogenic and can provoke potent immune responses in humans.

In the absence of safe and effective vaccines that induce a protective response against all wild and mutant strains, there is no realistic possibility of ending the COVID19 pandemic.[48] Some vaccines authorized for emergency use showed high efficacy against the primary variant of SARS-CoV-2. However, the reduced effectiveness of vaccines against VOCs has been reported.[49-52] Some cases also became infected with mutated strains of the virus after receiving a second dose of effective vaccines such as mRNA-1273 (Moderna) or BNT162b2 (Pfizer – BioNTech).[53] Previous studies indicated that surface glycoprotein has a crucial role in cell fusion by identifying and binding to the ACE2 receptor[9,54]; hence, mutations in spike antigen are involved in viral infectivity and transmissibility.[55,56] Therefore, to overcome these issues, we used mutant spike epitopes of five VOCs alongside conserved spike epitopes to design an efficient vaccine. Similarly, Khan et al developed a West Nile virus by attaching predicted B-cell, Th, and CTL epitopes with appropriate linkers.[57]

Cholera toxin subunit B (CTB) is a safe part of cholera toxin that can trigger B and T-cell immune responses. Guo et al constructed a multi-epitope *Helicobacter pylori* vaccine and included CTB as an adjuvant. According to the results, the developed vaccine was capable of eliciting a high level of antibody responses. So, in the current study, we attached CTB to the designed vaccine using the EAAAK linker to provoke immune responses.[58] To ensure the effectiveness of our vaccine, various analyzes were carried out. Physicochemical properties were predicted using ExPASy ProtParam, which indicated a molecular mass of 59.12 KD. The isoelectric point is a vital physicochemical parameter for the vaccine because at this point, the solubility and electrical repulsion are low, so the probability of aggregation and precipitation at this pH is high.[59] Our vaccine isoelectric point was 9.95, demonstrating that our vaccine is moderately basic. An index of protein instability less than 40 indicates protein stability; our vaccine instability index was 31.82, which proves that the vaccine is stable. The GRAVY of the final vaccine was -0.468, indicating that our vaccine is hydrophilic. Recently Ullah et al utilized the ExPASy ProtParam server to assess the physicochemical properties of their subunit vaccines against the Ebola virus.[60] Another important property of a vaccine is population coverage. The subject of population coverage is attributed to the MHC polymorphism, and it has shown that diverse HLA types are expressed at dramatically varying frequencies in different regions. Forasmuch as genetic diversity in the

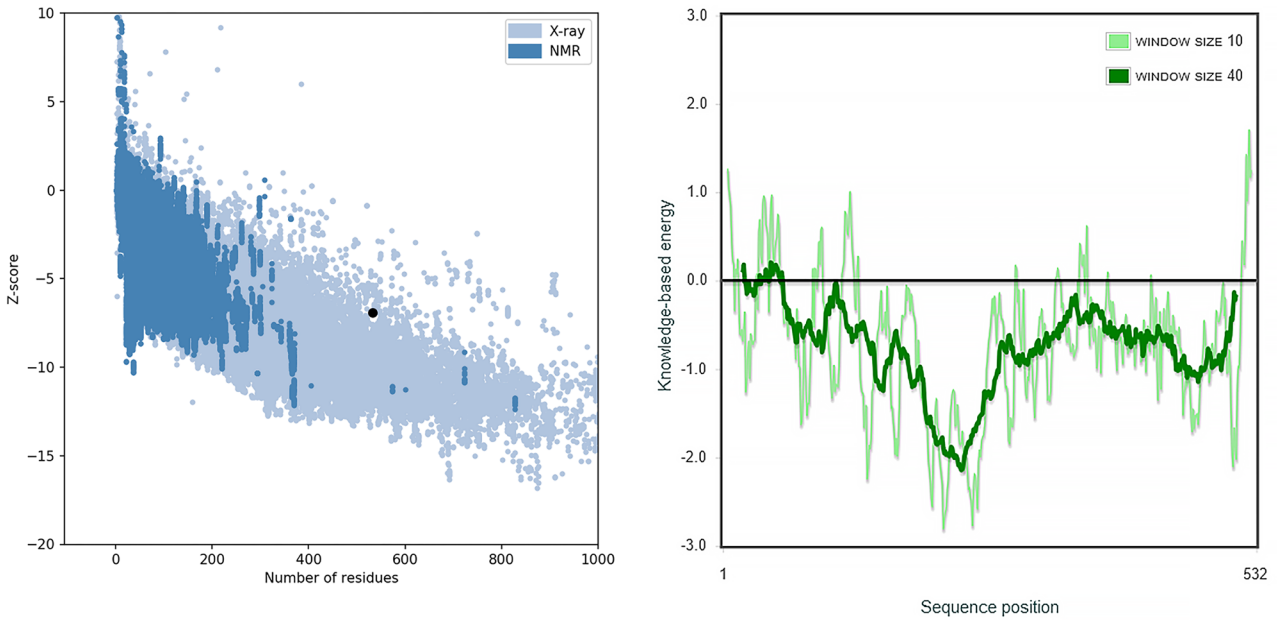

Figure 5. Vaccine 3D structural validation using ProSA. The refined vaccine calculated a Z-score value of -6.94 indicating an appropriate overall model quality (a). No error was found in the vaccine residue score and local model quality (b). Positive values are related to inaccurate regions of the vaccine structure

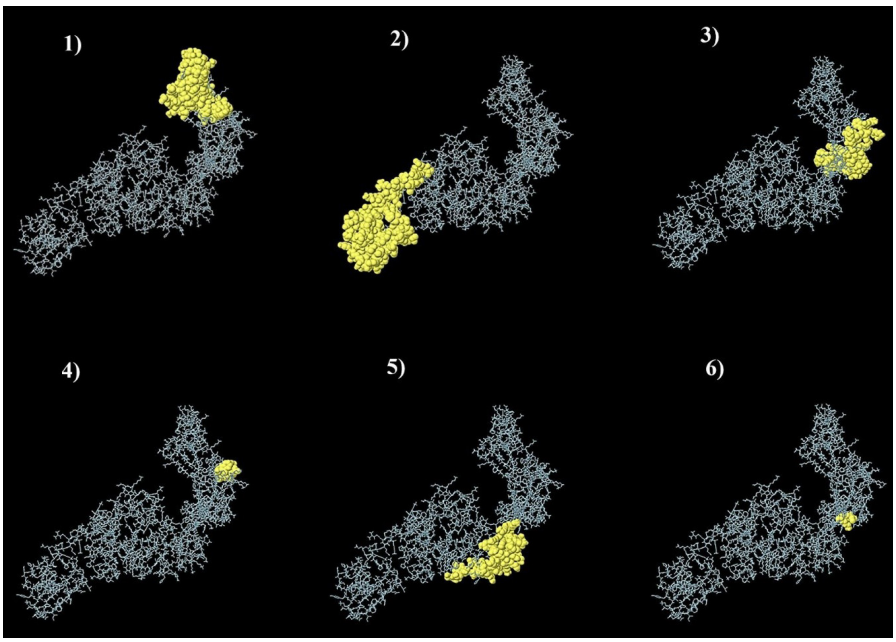

Figure 6. Conformational B-cell epitopes 3D structures. Six B-cell epitopes were presented by the ElliPro server. The final vaccine is shown in the skeletal model and discontinuous. Epitopes are shown in yellow

Table 9. Residue number and scores related to conformational B-cell epitopes

No.	Score	Number of residues
1	0.789	64
2	0.733	97
3	0.709	57
4	0.628	13
5	0.6	46
6	0.532	3

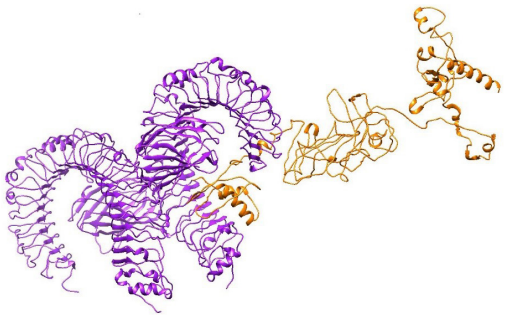

Figure 7. Vaccine -TLR docked complex. Chosen docking complex had the lowest energy score of -1186.6. TLR and vaccine are shown on the left (purple) and right (orange) respectively

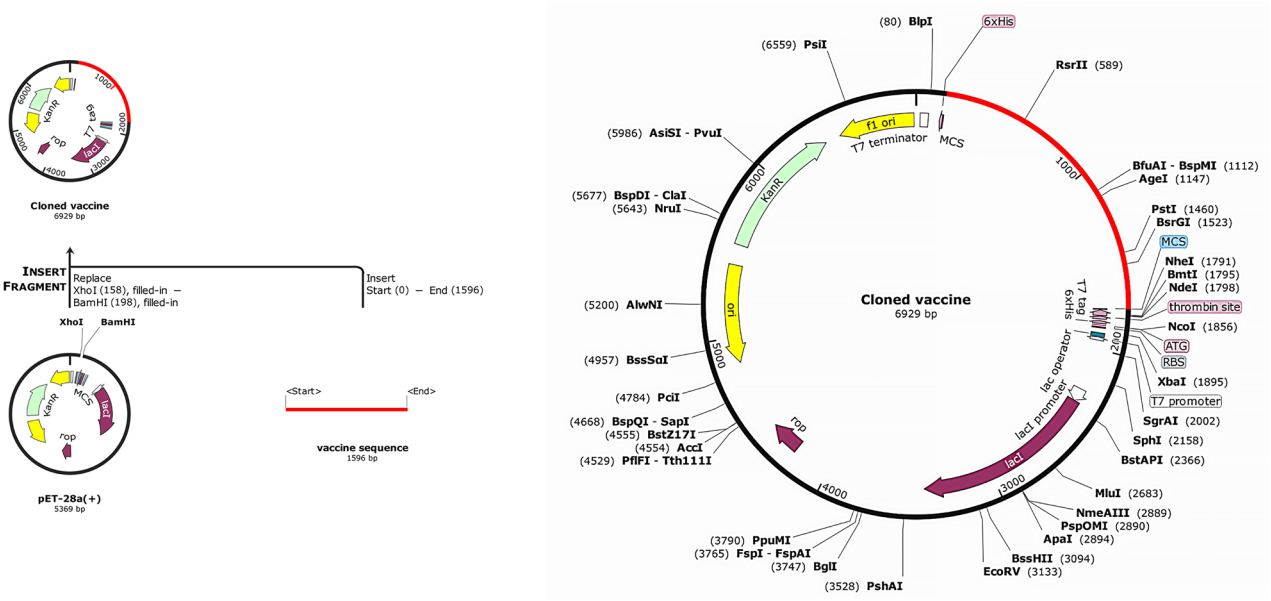

Figure 8. Vaccine sequence in silico cloning. The optimized sequence of the vaccine was inserted into the Pet-28a (+) between BamH1 and Xho1 restriction sites (a) and the final cloned vaccine total length was 6929 bp (b). The vaccine sequence is shown in red

host may affect vaccine efficacy, predicting population coverage is a critical step before starting costly clinical trials.[24,61] Our vaccine world coverage was 93.03% and in regions with the highest infection and death rates, including North America, Asia, South America, and Europe, it was over 80%. Also, the highest coverage was observed in Sweden (99.15), Southeast Ireland (99.32), Northern Ireland (99.08), England (99.0), Finland (98.65), and Germany (98.69). These results indicate a high population coverage of the designed vaccine.[24] A similar method was employed by Shi et al for a vaccine designed against MERS-CoV.[62]

Conclusion

In conclusion, the vaccine designed in this study by immunoinformatics tools and computer-based approaches showed specific reactivity with Toll-like immunoreceptor. Given the validation of other epitope-based vaccines in in-vivo and in-vitro studies,[63,64] our vaccine might be capable of providing strong immunity in a wide range of populations.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability Statement

Not applicable.

Ethical Approval

This study was approved by the Ethical Committee of Tabriz University of Medical Sciences, Tabriz (IR.TBZMED.VCR.REC.1399.385).

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Supplementary Files

Supplementary file 1. Sequence alignment of different spike glycoproteins.

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