Original Article
DOI: https://doi.org/10.70749/ijbr.v3i6.1476

INDUS JOURNAL OF BIOSCIENCE RESEARCH
https://induspublishers.com/IJBR
ISSN: 2960-2793/ 2960-2807

Computational Approaches to Design Cytotoxic Lymphocyte (CTL) Epitope-Based Vaccine Targeting the Spike (S) Protein of SARS-CoV-2

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ARTICLE INFO

Keywords: Multiepitopes, SARS-CoV-2, cytotoxic lymphocyte (CTL), AAY Linker, Molecular Docking, pET-28b.

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Declaration
Authors' Contribution
All authors equally contributed to the study and approved the final manuscript

Conflict of Interest: No conflict of interest.

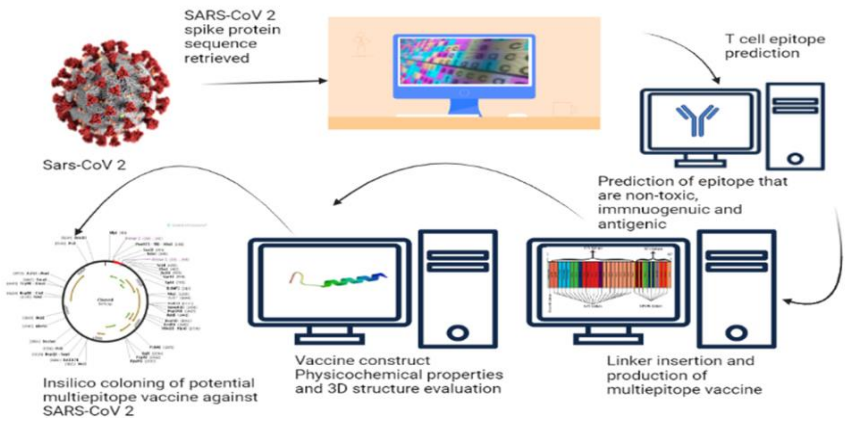
Funding: No funding received by the authors.

Article History
Received: 07-03-2025 Revised: 26-05-2025
Accepted: 04-06-2025 Published: 16-06-2025

ABSTRACT

The ongoing global threat posed by SARS-CoV-2 necessitates the rapid development of effective vaccines. This study employed a computational pipeline to design a multi-epitope vaccine targeting the spike (S) glycoprotein of SARS-CoV-2. Cytotoxic T lymphocyte (CTL) epitopes were predicted using immunoinformatics tools and were screened based on their non-toxicity, immunogenicity, and antigenicity. High-affinity epitopes were sequentially linked via AAY linkers to construct a rationally designed vaccine candidate. The tertiary structure of the construct was modeled and evaluated for structural stability and desirable physicochemical properties. To assess immunogenic potential, molecular docking was performed with key immune receptors, including Toll-like receptor 3 (TLR3) and major histocompatibility complex class I (MHC-I), demonstrating strong and specific binding interactions. Furthermore, the vaccine gene was codon-optimized and in silico cloned into the pET-28b(+) expression vector, yielding a construct of 5476 base pairs. The collective in silico findings support the designed multi-epitope vaccine as a promising candidate capable of inducing robust and long-lasting cell-mediated immunity against SARS-CoV-2. Experimental validation is warranted to confirm these computational predictions.

Graphical Abstract



INTRODUCTION

The rapid increase in human population and migration has led to the emergence of metropolitan nations, which contribute not only to ecological disturbances and climate change but also to the global spread of infectious diseases that pose significant risks to public health [1]. Humanity has faced numerous bacterial and viral diseases, ranging from those with minimal impact to others with catastrophic consequences. Before the 21st century, coronaviruses were primarily recognized as one of several etiological agents of the common cold in otherwise healthy individuals [2]. A significant number of pneumonia cases have been reported in Wuhan, China, caused by a disease subsequently named coronavirus disease 2019 (COVID-19). Following the sequencing of the pathogen's complete genome, it was identified as a novel coronavirus,

designated SARS-CoV-2. This virus is responsible for severe respiratory illnesses, including pneumonia and acute respiratory distress syndrome (ARDS) [3, 4].

Moreover, SARS-CoV-2 primarily spreads through respiratory aerosols and direct contact between individuals. The COVID-19 pandemic has rapidly escalated, resulting in millions of confirmed deaths worldwide. Older adults with underlying comorbidities have been identified as particularly vulnerable to severe outcomes from this deadly disease. In MERS, SARS-CoV-2, and SARS-related disorders, lower respiratory diseases such as bronchiolitis, bronchitis, and pneumonia are frequent [5]. Coronaviruses have an enclosed structure with a positive-sense RNA genome between 25 and 32 kbp. They have already been found in many hosts, mostly animals, including civets, camels, pigs, dogs, cats, bats, and bats [6, 7]. Cough, fever, dyspnea, and sporadic diarrhea were the SARS symptoms most often experienced. In 2012, Saudi Arabia was where the MERS-CoV was first identified [8]. Renal failure, digestive issues, and atypical pneumonia were the symptoms. Four distinct structural proteins, including S, M, N, and E, and many non-structural proteins known as nbsp, are found in the coronavirus genome, which comprises about 30,000 nucleotides [9-12]. The 2′-OMTase (nsp16) is a protein needed for viral replication and expression in host cells [13].

One open reading frame for a polyprotein with amino acids (9,860) and two un-translated sequences at each of its flanking ends make up the RNA genome of the newly discovered SARS-CoV2. Inside the genome, beginning with 5' replicase, the structural proteins S, E, and N at the N-terminal are organized [14]. The spike protein is considered to be involved in both viral entry into host cells and viral transmission. It performs a variety of molecular machinery tasks. The S1 subunit domain first contacts the host cell-surface receptor, and the host and viral cell membranes are fused by the S2 subunit domain [15]. Since the S1 subunit of the S protein's RBD (receptor binding domain) binds to the ACE2 receptor on host cells, [16] specific antibodies created by immunizing RBD could successfully prevent the viral attack by disrupting the contact. Consequently, SARS-CoV and MERS-CoV vaccines have been developed using this domain [17].

A study revealed that the receptor-binding domain (RBD) of SARS-CoV-2 has a higher affinity for the human ACE2 receptor than that of SARS-CoV, primarily due to mutations in key residues. Additionally, the SARS-CoV-2 RBD did not cross-react with antibodies targeting the SARS-CoV RBD, indicating notable antigenic differences. These findings underscore the need for further research to develop effective therapeutics and vaccines specifically targeting SARS-CoV-2 [18].

For instance, toll-like receptor 4 (TLR4) has been implicated in the pathophysiology of COVID-19. It is hypothesized that SARS-CoV-2 interacts with and activates TLR4, leading to upregulation of ACE2 expression, which facilitates viral entry. Moreover, inhibition of TLR4 has been shown to reduce the viral infection burden, highlighting its potential as a therapeutic target [19-22]. The strong interaction between the SARS-CoV-2 spike protein and TLR4 promotes upregulation of ACE2 expression, thereby facilitating viral entry into host cells. Developing a spike protein-based antigenic vaccine that targets TLR4 could be crucial in preventing the binding of the viral spike protein, ultimately helping to regulate ACE2 expression and inhibit SARS-CoV-2 infection. According to prior research [23], inhibiting TLR4 would lessen viral infection.

The selected T-cell epitopes engage T-cell receptors, proliferating and orchestrating essential immune responses. Consequently, inclusion of T-cell epitopes is critical for the design of both $CD8^+$- and $CD4^+$-dependent vaccines against lethal pathogens. Specifically, $CD4^+$-based vaccines target exogenous antigens that are internalized by antigen-presenting cells (APCs), processed within the endosomal pathway, loaded onto MHC class II molecules, and presented to $CD4^+$ T helper cells. In contrast, $CD8^+$-based vaccines deliver endogenous antigens that are degraded by the proteasome, translocated into the endoplasmic reticulum, loaded onto MHC class I molecules, and presented to $CD8^+$ cytotoxic T cells [16, 24, 25]. Multi-epitope vaccines (MEVs) offer significant advantages over single-epitope vaccines due to their enhanced structural stability, target specificity, and the ability to induce broader immune responses, making them more cost-effective and time-efficient [26].

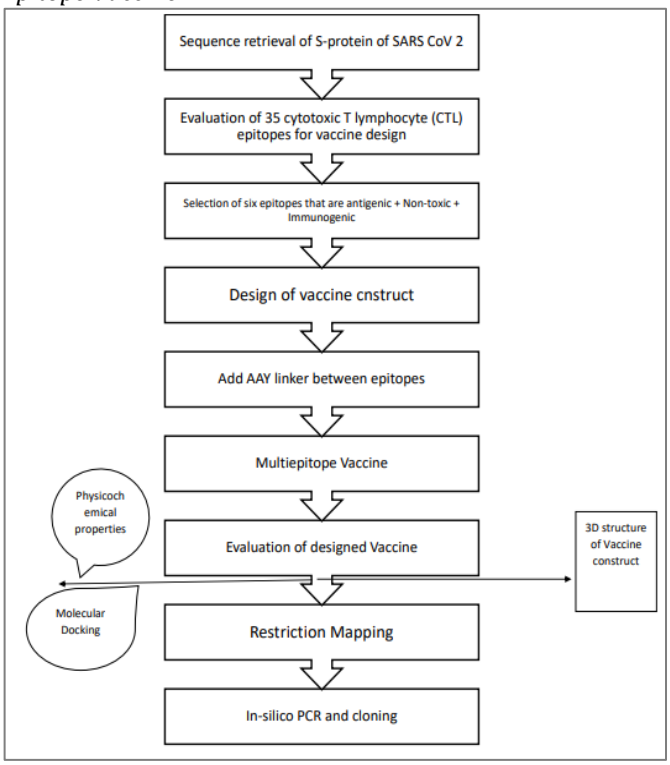
In this study, we designed multi-epitope vaccines (MEVs) targeting the SARS-CoV-2 spike protein using a comprehensive immunoinformatics approach. Major histocompatibility complex class I (MHC-I) alleles were analyzed to predict cytotoxic T lymphocyte (CTL) epitopes from the spike protein. A total of 35 CTL epitopes were evaluated for toxicity, antigenicity, and immunogenicity. Six epitopes that were non-toxic, antigenic, and immunogenic were selected and joined using AAY linkers to enhance immunogenic potential, resulting in the construction of the final multi-epitope vaccine sequence. Structural modeling and in silico analysis were conducted to assess the physicochemical properties, structural stability, and immunogenicity of the MEV construct. Molecular docking studies demonstrated strong binding affinity and stable interactions between the MEV and key human immune receptors. Codon optimization was performed for expression in the *Escherichia coli* (E. coli) system, and in silico cloning was carried out using the pET-28b(+) expression vector to validate the expression potential of the vaccine construct.

MATERIALS AND METHODOLOGY

Figure 1 shows the flow chart illustration of complete sequence of steps from SARS-CoV-2 Spike (S) protein sequence retrieval to molecular docking between predicted epitopes and their corresponding receptors.

Figure 1

Flow Chart Briefing of In-Silico Methodology for Multi Epitope Vaccine.



S-protein Sequences Retrieving

The sequence of spike regions in COVID-19 was retrieved using NCBI (National Center for Biotechnology Information). There are 1273 amino acids in protein. (https://www.ncbi.nlm.nih.gov/protein/QZH77230.1/) under Accession # >QZH77230.1. We selected the FASTA data type for standard sequence representation.

Evaluation of Cytotoxic T lymphocytes (CTL) Epitopes

To manufacture a subunit vaccine, epitope identification for cytotoxic T lymphocytes (CTL) is crucial. The CTL identifies pathogens by binding to specific CTL epitopes on the MHC class I molecules [27]. NetCTL1.2 server (https://services.healthtech.dtu.dk/service.php?NetCTL-1.2) was used to predict potential CTL peptides for the COVID-19 surface S-proteins. We pasted the selected protein's FASTA sequence into the query box and set the parameters by default [28].

Prediction of Toxic/nontoxic Nature of Epitopes

To determine how the vaccine interacted with the host body environment [29], TonxinPred was used (https://webs.iiitd.edu.in/raghava/toxinpred/). It is an SVM model to identify molecules as hazardous or nontoxic. Only nontoxic peptides were selected by entering the FASTA-formatted sequence of those founded epitopes into the search query [30].

Antigenicity Evaluation

The antigenicity of nontoxic epitopes was evaluated by the Vaxijen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) by a standard value of 0.4 [31]. It is based on the auto cross-covariance (ACC) transformation of protein sequences into uniform vectors of principal amino acid properties. These epitopes were tested by pasting their sequences into a query box. A virus model was chosen, and the threshold value was fixed at 0.4. As a result, only 11 of the 33 nontoxic epitopes were expected to be immunogenic.

MHC-I Immunogenicity Analysis

The T-cell epitope must be immunogenic to activate CD8+ or CD4+ T cells. The IEDB tool(http://tools.iedb.org/immunogenicity/) for MHC-I immunogenicity assessment was used [32]. On the IEBD server, the conversancy evaluation was carried out [33]. The default settings were used to predict immunogenicity, and peptides with positive values were picked for further research [34]. The sequence of the peptides must be maintained throughout all identified variation sequences to develop broad-spectrum peptide-based vaccines [35].

Construction of Multi-epitope Vaccine Candidate Sequence

The highly antigenic, nontoxic, and immunogenic epitopes were chosen for the final vaccine construct. They were used to construct multi-epitope vaccine sequences. An AAY linker linked the linear CTL epitopes. Linkers were used to enhance their immunogenicity.

Evaluation of Multi-epitope of Vaccine Constructs Physicochemical Properties

We explored the bioinformatics tool Protparam (https://web.expasy.org/cgi-bin/protparam/protparam) to evaluate the physiochemical characteristics of multi-epitopes in the final vaccine construct. The computed parameters consisted of theoretical pI, extinction coefficient, amino acid composition, instability index, molecular weight, instability and aliphatic index, atomic composition, estimated half-life, and grand average of hydropathicity (GRAVY).

Tertiary Structure or 3D Structure Evaluation of Protein

The structure prediction tool I-TASSER (Iterative Treading ASSEmbly Refinement) server (https://zhanggroup.org/I-TASSER/) was used to generate the tertiary or three-dimensional (3D) model of the multi-epitope vaccine. This integrated platform for computational protein complexes and function analysis identifies similar structural patterns in the Protein Data Bank, depending on the sequence-to-structure-to-function strategy [36]. A model with a T.M. value > 0.5 has precise topologies and a T.M. value.

Molecular Docking of Multi-epitope Vaccine Construct

Different cytokines and interferons are released from Toll-like receptors TLRs to eradicate the viruses. The binding affinity of our vaccine construct to MHC1 will activate adaptive immunity. The multi-epitope vaccine construct was docked with Toll-like receptor 3 (TLR3) and MHC1 by using Boston University online server Cluspro (https://cluspro.bu.edu/home.php) was used for this analysis [37], and Pymol (https://pymol.org/2/) was used for the visualization of docking complex.

Restriction Mapping of Multi-epitope Vaccine Construct

NEBCutter 2.0 (http://nc2.neb.com/NEBcutter2/) is also used to find all possible restriction sites. By pasting the

sequence (text or fasta format) into the query box with default settings, we get the list of enzymes that can digest the sequence. An appropriate expression vector was chosen by SnapGene 1.1.3 online tool.

Primer Designing and Vector Selection

Primer design for the multi-epitope vaccine construct was performed using SnapGene (version 1.1.3; https://www.snapgene.com/release-notes-old-blog-version/version-1-1-3/), enabling the development of highly specific and sensitive primers prior to initiating costly laboratory assays. The pET-28b(+) expression vector was chosen for in silico cloning due to its suitability for high-level protein expression in *Escherichia coli*.

In Silico PCR and Cloning Multi-epitope Vaccine Construct

The in-silico polymerase chain reaction was used to analyze the consequences of selective amplification of the target genetic region for the theoretical possibility of a successful PCR. It was used to determine the prime location, orientation, and binding efficiency and calculate their Tms. The DNA sequence was cloned into a pET-28b vector between PaeR71-Tli1-XhoI and NdeI 1 restriction sites. These restriction sites were not present at both ends or within the DNA sequence of the vaccine construct. Therefore, the restriction sites NdeI and XhoI were added to the N and C terminals of the optimized complementary DNA sequences. After this, the insert was treated with these two restriction enzymes in silico in SnapGene v3.3.4, and the insert DNA was successfully cloned into the vector. The vector, along with the insert, was 5476 base pairs in length.

RESULTS

Evaluation of Cytotoxic T Lymphocytes (CTL) Epitopes in Spike (S) Protein Sequence

Using the NetCTL 1.2 website server fixed at the standard value for epitope records, 35 CTL epitopes for the S protein were found, shown in **Table 1**.

Table 1

Cytotoxic T Lymphocytes epitopes of SARS-CoV-2 Spike protein.

Protein	Cytotoxic T lymphocytes (CTL) epitopes
Spike protein of SARS-CoV-2	NSFTRGVYY
//	STQDLFLPF
//	VLPFNDGVY
//	CNDPFLDVY
//	WMESXXXVY
//	YSSANNCTF
//	SANNCTFEY
//	FVFKNIDGY
//	NIDGYFKIY
//	WTAGAAAYY
//	GAAAYYVGY
//	ITDAVDCAL
//	LSETKCTLK
//	NATRFASVY
//	RISNCVADY
//	CVADYSVLY
//	NSASFSTFK
//	ASFSTFKCY
//	FTNVYADSF
//	ERDISTEIY
//	TSNQVAVLY
//	QLTPTWRVY
//	GAEHVNNSY
//	VASQSIIAY
//	STECSNLLL
//	ECSNLLLQY
//	RSFIEDLLF
//	LTDEMIAQY
//	GTITSGWTF
//	RVDFCGKGY
//	FVSNGTHWF
//	VSNGTHWFV
//	VLKGVKLHY

Assessment of the Toxic/nontoxic nature of epitopes

Additionally, toxicity assessments of the 35 S area of SARS-CoV-2 epitopes were performed. **Table 2** shows the Evaluation of Nontoxic epitopes for vaccine construction. Based on the results of the ToxinPred tools, only two epitopes were identified as hazardous. Additional antigenicity and immunogenicity tests were conducted on the 33 nontoxic epitopes that remained.

Table 2

Evaluation of Nontoxic epitopes for vaccine construct

Peptide sequence	SVM score	Evaluation	Charge	Molecular weight	Hydrophobicity	Hydropathicity	Hydrophilicity
NSFTRGVYY	-0.73	Non-Toxin	1.00	1106.32	-0.17	-0.61	-0.61
STQDLFLPF	-1.49	Non-Toxin	-1.00	1067.33	0.04	0.34	-0.61
VLPFNDGVY	-1.29	Non-Toxin	-1.00	1023.28	0.11	0.52	-0.71
CNDPFLDVY	-0.68	Non-Toxin	-2.00	1085.31	-0.05	-0.01	-0.32
WMESVY	-1.15	Non-Toxin	-1.00	814.00	0.05	-0.07	-0.87
YSSANNCTF	-0.48	Non-Toxin	0.00	1006.16	-0.12	-0.39	-0.63
SANNCTFEY	-0.49	Non-Toxin	-1.00	1048.20	-0.16	-0.69	-0.33
FVFKNIDGY	-0.75	Non-Toxin	0.00	1102.38	0.02	0.19	-0.49
NIDGYFKIY	-1.13	Non-Toxin	0.00	1132.41	-0.02	-0.23	-0.50
WTAGAAAYY	-1.07	Non-Toxin	0.00	973.16	0.15	0.29	-1.16
GAAAYYVGY	-1.17	Non-Toxin	0.00	934.13	0.19	0.54	-1.10
ITDAVDCAL	-0.67	Non-Toxin	-2.00	920.16	0.08	1.21	-0.17
LSETKCTLK	-1.22	Non-Toxin	1.00	1022.35	-0.38	0.43	0.43
NATRFASVY	-0.64	Non-Toxin	1.00	1028.24	-0.02	-0.47	-0.47
RISNCVADY	-0.78	Non-Toxin	0.00	1040.27	-0.07	-0.07	-0.07
CVADYSVLY	-0.52	Non-Toxin	-1.00	1032.29	1.07	-0.84	-0.84
NSASFSTFK	-1.04	Non-Toxin	1.00	988.17	-0.34	-0.20	-0.20
ASFSTFKCY	-0.66	Non-Toxin	1.00	1053.30	0.27	-0.62	-0.62
FTNVYADSF	-1.10	Non-Toxin	-1.00	1063.24	0.20	-0.69	-0.69
ERDISTEIY	-1.79	Non-Toxin	-2.00	1125.32	-0.98	0.67	0.67
TSNQVAVLY	-1.17	Non-Toxin	0.00	994.24	0.47	-0.81	-0.81
QLTPTWRVY	-1.50	Non-Toxin	1.00	1163.47	-0.58	-0.73	-0.73

GAEHVNNSY	-0.58	Non-Toxin	-0.50	990.12	-1.13	-0.12	-0.12
VASQSIIAY	-1.09	Non-Toxin	0.00	951.21	1.16	-0.84	-0.84
STECSNLLL	-0.52	Non-Toxin	-1.00	979.24	0.51	-0.33	-0.33
ECSNLLLQY	-0.39	Non-Toxin	-1.00	1082.37	0.14	-0.56	-0.56
RSFIEDLLF	-0.82	Non-Toxin	-1.00	1139.44	0.60	-0.12	-0.12
LTDEMIAQY	-0.52	Non-Toxin	-2.00	1083.35	-0.06	-0.21	-0.21
GTITSGWTF	-1.10	Non-Toxin	0.00	969.19	0.30	-0.96	-0.96
RVDFCGKGY	-0.34	Non-Toxin	1.00	1044.31	-0.50	0.19	0.19
FVSNGTHWF	-0.92	Non-Toxin	0.50	1094.32	0.03	-1.14	-1.14
VSNGTHWFV	-0.92	Non-Toxin	0.50	1046.28	0.19	-1.03	-1.03
VLKGVKLHY	-1.26	Non-Toxin	2.50	1056.46	0.37	-0.38	-0.38

Antigenicity Evaluation

The antigenicity of nontoxic epitopes was one of the essential conditions for creating a successful vaccination, done by the Vaxijen server at a default value of 0.4. Eleven peptides were shown to be antigenic, ranging in antigenic value from 0.5 to 1.2. **Table 3** shows the antigenic nature and score of nontoxic peptides.

Table 3

Antigenic nature and score of nontoxic peptides.

Sr. No	Peptide sequences	Antigenic nature	Antigenic score
1	STQDLFLPF	Possible ANTIGEN	0.6619
2	VLPFNDGVY	Possible ANTIGEN	0.4642
3	WTAGAAAYY	Possible ANTIGEN	0.6306
4	GAAAYYVGY	Possible ANTIGEN	0.6604
5	ITDAVDCAL	Possible ANTIGEN	0.5260
6	LSETKCTLK	Possible ANTIGEN	0.6883
7	TSNQVAVLY	Possible ANTIGEN	0.4387
8	QLTPTWRVY	Possible ANTIGEN	1.2119
9	GAEHVNNSY	Possible ANTIGEN	0.9347
10	STECSNLLL	Possible ANTIGEN	0.4871
11	VLKGVKLHY	Possible ANTIGEN	0.9364

Evaluation of Immunogenicity

Epitopes' capacity to stimulate T cells and cellular immunity will increase as their immunogenicity value rises. The IEBD tool showed only six immunogenic peptides with a positive score cutoff; those were selected to construct the vaccine. **Table 4** shows the final nontoxic, antigenic, and immunogenic epitopes.

Table 4

Number of epitopes that are Nontoxic, antigenic, and immunogenic

Sr. No	Epitopes	Immunogenicity score	Length
1	QLTPTWRVY	0.31555	9
2	VLPFNDGVY	0.1815	9
3	WTAGAAAYY	0.15259	9
4	GAAAYYVGY	0.09963	9
5	ITDAVDCAL	0.08501	9
6	STQDLFLPF	0.06828	9

Construction of multi-epitope subunit vaccine

AAY linkers specialized for CTL epitopes were placed between the chosen peptide sequences to increase immunogenicity. Small sequences of amino acids called "linkers" were naturally created to join various protein domains. These linkers are mostly inflexible.

Evaluation of physicochemical properties of the vaccine

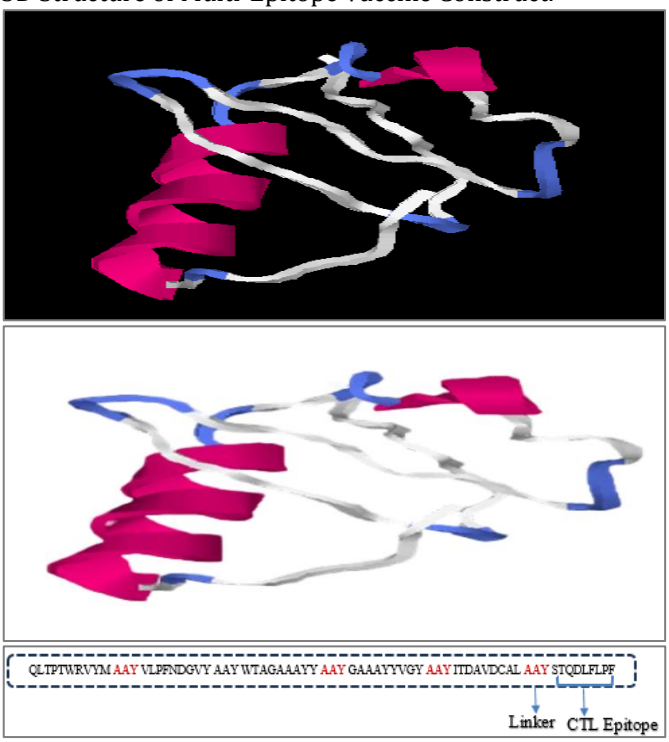
The multi-epitope vaccine design has a molecular weight and theoretical pI of 6615.43 and 3.77, respectively. Theoretical pI values have indicated the basic nature of the vaccine. The value of the extinction coefficient for the vaccine construct at 280 nm, where all cysteine (Cys) residues were reduced, was 24410 M-1 cm-1. One positively charged residue (Arg and Lys) and four negatively charged residues (Asp and Glu) are present. The protein is stable, as indicated by the instability index (II), which was calculated to be 18.57, as the protein is unstable if the instability index is greater than 40. The protein's aliphatic index, which was 86.50, shows that the vaccine is thermostable. The S (Ser) makes up the sequence's N-terminal. The estimated half-life for Escherichia coli in vivo is >10 hours for mammalian reticulocytes; in vitro, it is 1.9 hours; and for yeast in vivo, it is >20 hours. The multiple epitopes for the vaccine design are hydrophilic, as indicated by the Grand Average of Hydropathicity (GRAVY) of 0.463.

Prediction of the 3D structure of the protein

The structure prediction tool I-TASSER generated the multi-epitope vaccine's tertiary or three-dimensional (3D) model, shown in **Figure 2**.

Figure 2

3D Structure of Multi-Epitope Vaccine Construct.



The evaluation of immunogenicity is a critical aspect of vaccine development, as it assesses the capacity of epitopes to stimulate T cells and elicit cellular immunity. In this study, the IEBD tool was employed to identify immunogenic peptides, and only those with a positive score cutoff were selected for further analysis. The final nontoxic, antigenic, and immunogenic epitopes are presented in **Table 4**, which includes the immunogenicity

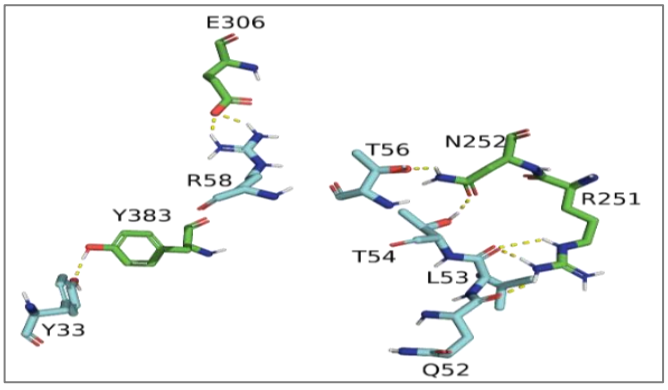
score and length of each epitope. To construct the multi-epitope subunit vaccine, AAY linkers specialized for CTL epitopes were strategically placed between the chosen peptide sequences. These linkers enhance the vaccine's immunogenicity by facilitating the proper folding and presentation of epitopes to the immune system. The use of small sequences of amino acids as linkers is a common approach in vaccine design, as they help maintain the structural integrity of the vaccine while ensuring flexibility for optimal epitope recognition by T cells. The physicochemical properties of the multi-epitope vaccine were evaluated to assess its stability and functionality. The vaccine construct has a molecular weight of 6615.43 and a theoretical pI of 3.77, indicating its relatively small size and essential nature. The extinction coefficient at 280 nm, where all cysteine residues were reduced, was calculated to be 24410 M^{-1} cm^{-1}, suggesting a significant presence of aromatic amino acids. The instability index of 18.57 and the aliphatic index of 86.50 indicate that the vaccine is stable and thermostable, respectively. The estimated half-life of the vaccine in various systems, such as Escherichia coli in vivo, mammalian reticulocytes in vitro, and yeast in vivo, further supports its stability. The Grand Average of Hydropathicity (GRAVY) score of 0.463 suggests that the multiple epitopes are hydrophilic, which can enhance solubility and interaction with immune components.

Molecular Docking with TLR3 and MHC1

After I-TASSER predicted the 3D structure of the vaccine, the structures of TLR3 and MHC1 (PDB IDs 2A0Z and 1I1Y, respectively) were taken from the Protein Data Bank (PDB). The lowest binding energy model among the Cluspro-generated dock complex models was chosen because a dock complex 2 with a lower energy score would be more stable. The multi-epitope vaccine design demonstrated the closest binding contract with the TLR3 receptor, which also had the lowest energy score (-901.2). The model chosen for investigation had the lowest energy score after docking with MHC1, which was -1014.8. The multi-epitope vaccine construct binds to the TLR3 receptor. A total of six hydrogen bonds were observed among active residues of the TLR3 receptor and multi-epitope vaccine constructs, i.e., E-306, Y-383, N-252, and R-251 from the receptor and R-58, Y-33, T-56, T-54, L-53, and Q-52 from the multi-epitope vaccine constructs, as shown in **Figure 3**.

Figure 3

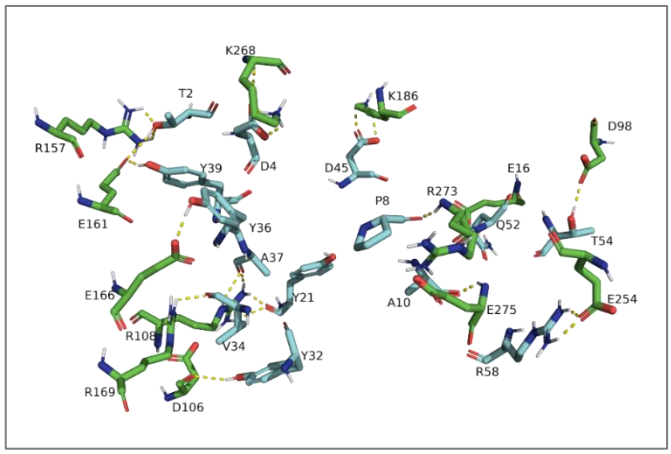
Molecular Docking between TLR3 and Multi-Epitope Vaccine Construct



The interaction between MHC1 and the multi-epitope vaccine construct involves fourteen hydrogen bonds. The MHC1 residues involved in the hydrogen bonds are R-157, E-161, E-166, R-108, R-169, D-106 K-268, R-273, E-16, E-275, E-254 and D-98. The multi-epitope vaccine construct residues involved in the hydrogen bonding to MHC1 are T-2, D-4, Y-36, Y-37, Y-39, A-37, Y-21, Y-32, D-45, P-8, A-10, R-58, and T-54 as shown in **Figure 4**.

Figure 4

Molecular Docking between MHC1 and Multi-Epitope Vaccine Construct

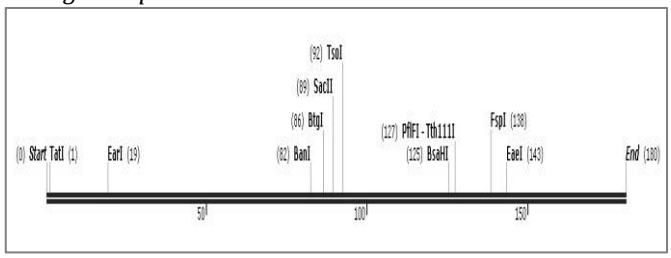


Restriction Mapping

After carefully considering the restriction locations, an acceptable expression vector was selected. All cleavage sites of the restriction enzyme are shown in **Figure 5.**

Figure 5

Restriction mapping of Multi Epitope Vaccine construct through Snap Gene

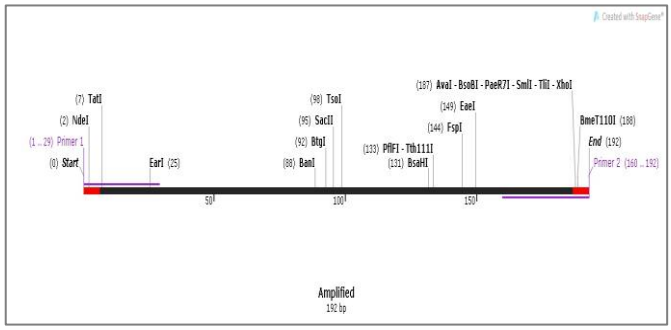


In-silico PCR and cloning

In silico PCR, the innovation of computational algorithms has allowed us to analyze the theoretical possibility of a successful PCR by designing highly specific and sensitive primers before starting expensive laboratory assays. In silico PCR results illustrated in **Figure 6**.

Figure 6

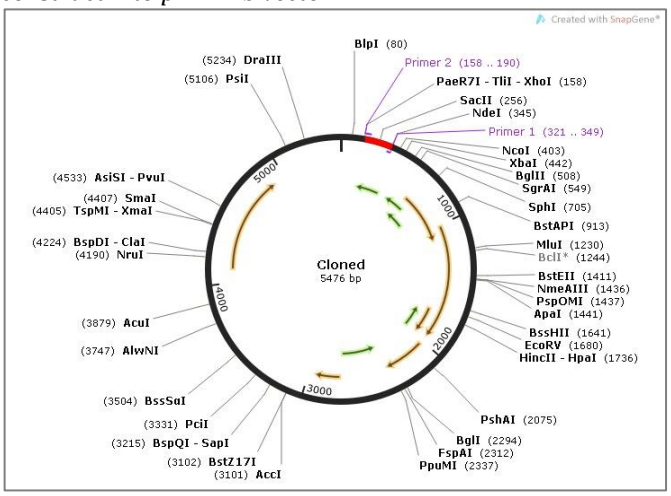
Amplified product of in-silico PCR of multi-epitope construct



In between the restriction sites PaeR71-Tli1-XhoI and Ndel1, a DNA sequence was cloned into the pET-28b vector. The Xho1 and Ndel-1 restriction sites were added at the N- and C- terminal ends, respectively. The **Figure 7**, the vector, along with the insert, is shown. Vaccine constriction is depicted in 'Red' color.

Figure 7

In silico restriction cloning of the multi-epitope for vaccine construct into pET-28b vector.



DISCUSSION

The emergence of coronavirus disease 2019 (COVID-19) has presented a significant challenge to global health due to its acute pneumonic infection and lack of specific preventive measures and targeted treatment options. The unique transmission and pathogenesis characteristics of COVID-19 have made it increasingly difficult to control the spread of this viral virus [38]. Traditional live or attenuated vaccines have shown a successful track record in response to the urgent need for effective preventive measures. However, their usage comes with concerns related to biosafety, including autoimmune or severe allergic reactions and challenges in synthesis and production. To address these issues, researchers have explored the development of entirely manufactured peptide-based vaccinations [39]. The study of COVID-19 anticipation has gained momentum with the release of fundamental information, including the whole genetic sequence of the virus. In this context, researchers have found some epitope regions that are very similar to other coronaviruses, like SARS-CoV, which has been well-studied in terms of epitope responses [40]. The spike glycoprotein, in particular, has been a focus of attention for its antigenic characteristics [41]. This information has led to the proposal of various epitope-based strategies, ranging from those targeting a single or a small number of viral proteins to those considering the entire viral proteome. These strategies aim to uncover the most effective epitomic regions in the spike proteins, further advancing our understanding in this area [42, 43].

Given the high infectivity and rapid transmission of SARS-CoV-2, a preventative vaccination is urgently required [44]. Previous research has demonstrated the feasibility and benefits of multi-peptide vaccines developed using bioinformatics techniques [45]. Ikram et al. (2018) point out that the study by Ojha et al. on a multi-epitope subunit vaccination against cancer caused by the Epstein-Barr virus using immune-informatics techniques shows the potential of this approach [45, 46]. Researchers have started making a multi-epitope subunit preventive vaccination for SARS-CoV-2 using immune-informatics techniques [47] because they now have information on the SARS-CoV-2 genome and proteome.

Immuno-informatics has emerged as a crucial discipline with a wide range of tools and datasets that aid in research allocation, facilitate the analysis of vast immunologic data obtained from experiments, and contribute to developing novel hypotheses. Its potential to improve and expand the field of immunology is evident, and bioinformatics methodologies and applications are being increasingly implemented in vaccine analytics to support various stages of preclinical, clinical, and post-licensure vaccine development [48]. Using bioinformatics methods, the spike protein of SARS-CoV-2 was utilized to identify cytotoxic T lymphocyte (CTL) epitopes. Subsequently, a multi-epitope vaccination was designed to induce both CD^{4+} and CD^{8+} T-cell immunological reactions [49]. While many studies have focused on vaccinations based solely on spike proteins, exploring other viral proteins as potential vaccine candidates is essential. This study chose eleven proteins with positive antigenicity for further epitope prognosis. After immunogenicity assessment using the IEDB bioinformatics program, it was determined that only six epitopes were immunogenic. While the precise immune mechanism remains fully understood, this technique provides valuable insights into possible SARS-CoV-2 antigens, ultimately guiding the selection of the SARS-CoV-2 protein for developing an efficient vaccine.

It was identified numerous protein epitopes for CD^{4+} and CD^{8+} T cells. Their protein list matched precisely with the findings of this study. It only identified T-cell epitopes; the present study focused on CTL epitopes to create a more potent vaccination. The T cell epitopes induced the development of effector T cells, immunological memory T cells, and IFN production through MHC class I molecule identification of internalized viral pathogens, which is crucial in defending against viral diseases [50]. The advancements in recombinant DNA technology have revolutionized various sectors, including biological and nutritional sciences, pharmaceuticals, and vaccine development. Pharmacological proteins and vaccine alternatives that were previously nonexistent have been designed, manufactured, and produced through these innovations [51]. The rise and development of bioinformatics have made it possible to test the effectiveness of new therapies and vaccines before they are put to the test in the lab [52].In this study, a multi-epitope protein made up of the major immunogens of the SARS-CoV-2 spike protein was made and cloned. It was done to help with the development of a candidate vaccine.

The urgent need for a reliable vaccination against COVID-19 is evident, particularly for underdeveloped nations where millions of individuals remain vulnerable to pathogenic COVID-19 species. To enhance the likelihood of protection against all pathogenic strains of COVID-19, the

study focused on the most immunogenic surface proteins of the virus, which were highly conserved in pathogenic strains. The proposed multi-epitope vaccine construct, evaluated through bioinformatics and immunoinformatics, exhibited promising interactions with the TLR3 receptor and MHC1.

Although the study presents a potential candidate vaccine against SARS-CoV-2, it is crucial to conduct further experiments to confirm its safety and effectiveness. The research successfully cloned a multi-epitope sequence for the final vaccine construct using the polymerase chain reaction (PCR) method and a T.A. cloning vector (pCR 2.1). The T.A. cloning method has several benefits, including effectiveness, economy, and routine application. Positive clones were identified through restriction digestion and PCR amplification.

This study provides valuable insights into developing a potential COVID-19 vaccine using immune-informatics and bioinformatics techniques. Exploring epitope-based strategies and multi-epitope vaccines offers promising avenues for overcoming challenges associated with traditional live or attenuated vaccines. Immuno-informatics has emerged as a vital discipline that facilitates research allocation, data analysis, and the development of novel hypotheses in the field of immunology. The study's findings contribute to the ongoing efforts to combat the COVID-19 pandemic and underscore the potential of bioinformatics in vaccine analytics and research. Nevertheless, further experimental validation is essential to establish the safety and efficacy of the proposed SARS-CoV-2 vaccine candidate. The continuous advancements in immune informatics and bioinformatics provide a powerful platform for accelerating vaccine research and development, offering hope for effectively managing infectious diseases.

CONCLUSION

This study utilized a comprehensive immunoinformatics-based approach to design a multi-epitope vaccine targeting the spike protein of SARS-CoV-2. Cytotoxic T lymphocyte (CTL) epitopes were carefully selected based on their predicted antigenicity, immunogenicity, and non-toxicity. These epitopes were joined using AAY linkers to enhance immunogenicity and construct a robust vaccine candidate. The predicted tertiary structure and favorable physicochemical properties of the construct support its stability and effectiveness. Molecular docking studies demonstrated strong binding affinities between the vaccine construct and key immune receptors, including TLR3 and MHC-I, indicating its potential to stimulate a sustained cell-mediated immune response. Additionally, in silico cloning into the pET-28b(+) expression vector (totaling 5476 bp) confirmed the feasibility of its expression in *E. coli*. The encouraging outcomes of this in silico investigation underscore the necessity for rigorous experimental validation. Preclinical evaluation in appropriate animal models will be essential to characterize the immunogenicity, protective efficacy, and safety profile of the multi-epitope vaccine construct. Subsequent clinical trials must then establish its tolerability and immunogenic potential in humans. Parallel efforts should continue to refine epitope selection and vaccine formulation, leveraging advances in bioinformatics and immunoinformatics to improve prediction accuracy and streamline development workflows. Integration of computational design with empirical data—such as high-throughput epitope mapping and structural vaccinology will further optimize antigen selection and enhance vaccine potency. Collaboration with regulatory agencies and biopharmaceutical partners is critical to translate these computational insights into scalable manufacturing processes. Ultimately, the convergence of in silico methodologies and experimental research holds promise not only for accelerating the development of SARS-CoV-2 vaccines but also for establishing a robust platform adaptable to future emerging pathogens.

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