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Original Article

In silico Design of a New Multi-epitope Polypeptide against *Vibrio cholerae O1 biovar ElTor*

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Abstract

Introduction: In this study, due to the importance of cholera as a water-borne infection, a new and appropriate multi-epitope polypeptide was designed against *Vibrio cholerae O1 biovar ElTor* using the reverse vaccinology method and immunoinformatic implements.

Materials and Methods: After identification of all ORFs of chromosome 1 bacterium, their outer membrane and secretory proteins were determined. Then, by removing the signal peptide and transmembrane domain, non-toxic, non-allergenic, and antigenic proteins were selected from the proteins of the previous step. Finally, high-score epitopes of the final antigenic proteins were identified using several specific software. To design the multi-epitope polypeptide, selected epitopes, and an appropriate adjuvant were connected using a flexible linker.

Results: To validate the biochemical properties of the designed polypeptide, we implemented a range of servers to evaluate the biochemical and physicochemical properties of the designed polypeptide. Subsequently, we performed a molecular binding study to investigate the interaction of the designed polypeptide with the MHC I and MHC II molecules. The designed polypeptide contained 277 amino acids with a half-life of approximately 30 hours in mammalian cells. The results confirmed that this designed polypeptide not only has sufficient antigenic properties without toxicity and allergenicity but also has a good affinity for binding to MHC molecules.

Conclusions: After synthesis and appropriate immunological tests, the designed polypeptide can be considered as a new candidate vaccine against *Vibrio cholera* to prevent the spread of infection.

Keywords: Cholera Infection, Epitope-based Polypeptide, Immunoinformatic, Molecular Docking, Reverse Vaccinology

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Introduction

Nonetheless, there are some approved vaccines against cholera infection, and a significant number of them are live-attenuated or inactivated oral.[1,2] Since each generation of vaccines has its drawbacks, current improvements in bioinformatics might lead to an advancement in vaccinology methods.[3] Since previous methods in vaccinology have not succeeded in attaining an effective vaccine against bacterial meningitidis, Rapouli used the reverse vaccinology (RV) approach for the first time against bacterial meningitidis.[4] Today, the RV method is used widely for vaccine design and development projects, and subsequently, various servers and databases have been expanded for this aim.[5,6] A number of these servers predict immunogenic proteins and epitopes of the target organism's genomic information by using various algorithms and epitope prediction methods.[7-11] Epitopes are regions of the protein sequence that possess immunogenic characteristics that could expose the host immune system. According to the definition of vaccine, it appears that the predicted epitopes would act the role of the target organism for the immune system. In the end, the produced immune responses against the epitopes might be able to immunize the host against the target pathogen.

In this study, we designed a multi-epitope polypeptide sequence to be used as a vaccine candidate against the *Vibrio cholera O1*. Considering the requirements to achieve a proper vaccine candidate, the highest score epitopes were selected, and accordingly, connected by using appropriate linkers to assemble a peptide sequence. To this aim, we investigated the multi-epitope polypeptide by several bioinformatics-based methods to predict the peptide features. Furthermore, molecular docking studies were performed to validate the affinity of the designed polypeptide with the major histocompatibility complex (MHC I and II) molecules.

Materials and Methods

Primary Data Collection and Prediction of ORFs and Proteins Positions

Firstly, the complete sequence of chromosome I of *V. cholerae O1 biovar ElTor* str. N16961 (NC-002505.1) was obtained from the nucleotide database of NCBI in FASTA format. After that, possible open reading frames (ORFs) of the sequence were identified and translated using the ORF

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finder tool (www.ncbi.nlm.nih.gov\ORFfinder). The PSORTb v3.0.3, an online web server with an accuracy of 96.5% (https://www.psort.org/psortb), was used for investigating the protein's positions.[12] Suggested outer membrane and extracellular proteins from the PSRORTb server were selected for further investigations. Then, the signal peptide and transmembrane domain of selected proteins are detected and removed by using the online web server SignalP-5.0 (https://services.healthtech.dtu.dk/services/SignalP-5.0/),[13] and TMHMM-2.0 server (https://services.healthtech.dtu.dk/services/TMHMM-2.0/),[14] respectively. The toxigenic, allergenic and antigenic properties of the selected proteins were evaluated by the ToxinPred2 server (https://webs.iiitd.edu.in/raghava/toxinpred2/batch.html),[15] AlgPred-2.0 server (https://webs.iiitd.edu.in/raghava/algpred/submission.html),[16] and VaxiJen-2.0 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen.html),[17] respectively. Afterward, the BLASTP program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the homology between selected proteins and human and mouse protein sequences. Also, the 3D structures of the MHC I and MHC II proteins were obtained in pdb format from the protein data bank (PDB).

Epitope Prediction and Multi-epitope Polypeptide Construction

To predict linear and conformational epitopes, sequences of the selected proteins are used for epitope mapping. The various methods including BepiPred-2.0 (tools.iedb.org/bcell),[18] LBtope (webs.iiitd.edu.in/raghava/lbtope/),[19] and ABCpred (webs.iiitd.edu.in/raghava/abcpred/),[20] were used to predict linear epitopes and ElliPro (tools.iedb.org/ellipro),[21] Discotope (tools.iedb.org/discotope),[22] and CBtope (webs.iiitd.edu.in/raghava/cbtope)[23] tools were used to predict conformational epitopes. Various features such as flexibility, surface accessibility, antigenicity, and hydrophilicity were considered in the selection of epitopes. In continuing, high-score B cell epitopes were selected from the predicted epitopes. Also, considering the importance of T cell epitopes in immunization by stimulating cellular immunity, selected B cell epitopes were used for T cell epitope mapping. The toxigenic, allergenic, and antigenic properties of the selected epitopes were evaluated by the ToxinPred2 server, AlgPred-2.0 server, and VaxiJen-2.0 server, respectively. Finally, the amino acid sequences of multiple predicted epitopes were linked together using flexible peptide linker GGGGS to generate the antigenic polytope. Finally, to construct the expression cassette in an expression plasmid, the final polypeptide sequence was converted into a nucleotide sequence using the bioinformatics database and the nucleotide sequence was used to simulate the restriction cloning into pET23a(+) plasmid using the SnapGene offline software.

Evaluation of the Designed Polypeptide

First, the surface accessibility, antigenicity, hydrophilicity, and flexibility of the designed polypeptide were predicted by the IEDB B cell epitope prediction tool (tools.iedb.org/bcell). Then, the toxigenic, allergenic, and antigenic properties of the designed polypeptide were evaluated by the ToxinPred2 server, AlgPred-2.0 server, and VaxiJen-2.0 server, respectively. Also, physicochemical properties of the designed polypeptide including the molecular weight, iso-electric point, *in vivo* half-life, net charge at pH=7, and water solubility were predicted using the Expasy Protparam tool (https://web.expasy.org/protparam/),[24] and the Pepcalc database (https://pepcalc.com).[25] The secondary structure of the designed polypeptide predicted by the Prabi server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html) through GOR IV secondary structure prediction method.[26] Subsequently, the three-dimensional structure of the protein was predicted by the I-TASSER web server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/).[27,28]

This server predicted a protein sequence's three-dimensional structure based on the structural templates of pdb models. In this study, model refinement was performed using the GalaxyWeb server (https://galaxy.seoklab.org/).[29,30] The geometric quality assessment was performed based on the Ramachandran plot (https://swift.cmbi.umcn.nl/servers/html/ramaplot.html).[31] Finally, the overall and local quality score for selected model were calculated using the ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php).[32] The molecular docking study was performed to validate the probability of coupling interaction between the designed polypeptide and MHC I and II molecules. The docking process was performed by using the HDOCK server for the evaluation of the binding energy between the designed polypeptide and MHC molecules.[33]

Results

Prediction of ORFs and Proteins Positions and Epitopes

In this study, 19470 ORFs were predicted from chromosome 1 of *V. cholerae O1 biovar ElTor*. Among them, the number of the outer membrane and secretory proteins was about 123 and 713 proteins, respectively. After removing the signal peptide and transmembrane domain of selected proteins, and selecting non-toxic and non-allergenic proteins, four antigenic proteins were selected. In the end, the seven high-score epitopes were identified from the final antigenic proteins (Table 1).

Construction of Multi-epitope Polypeptide

To design a multi-epitope polypeptide, 7 selected epitopes and 1 appropriate adjuvant from the cholera enterotoxin binding subunit CtxB with protein ID "WP_000593522.1" were linked together using a flexible linker. To facilitate the purification of the recombinant protein after expression, a histidine sequence was placed at the carboxyl terminus of the multi-epitope protein. Finally, cloning of the designed

Table 1. The Final Epitopes and Adjuvant Used in this Study

	Sequences	Toxin prediction (ToxinPred2)	The protective allergen (AlgPred-2.0)	Antigen (VaxiJen-2.0)
Epitopes	GQNANFSSESAKD	Non-Toxin	Probable Non-allergen	Antigen (1.2210)
	NGIGFTSNSYQL	Non-Toxin	Probable Non-allergen	Antigen (0.7575)
	GHTDSTGSDT	Non-Toxin	Probable Non-allergen	Antigen (2.9950)
	FSYQDWQSRDQRI	Non-Toxin	Probable Non-allergen	Antigen (1.1206)
	SKGQNSDQCDGSA	Non-Toxin	Probable Non-allergen	Antigen (1.7822)
	HTDSTGSDTTNQV	Non-Toxin	Probable Non-allergen	Antigen (1.8934)
	EGHTDSTGSDT	Non-Toxin	Probable Non-allergen	Antigen (2.9240)
Adjuvant	AGKREMAIITFKNGATFQVEVPG	Non-Toxin	Probable Non-allergen	Antigen (0.8226)

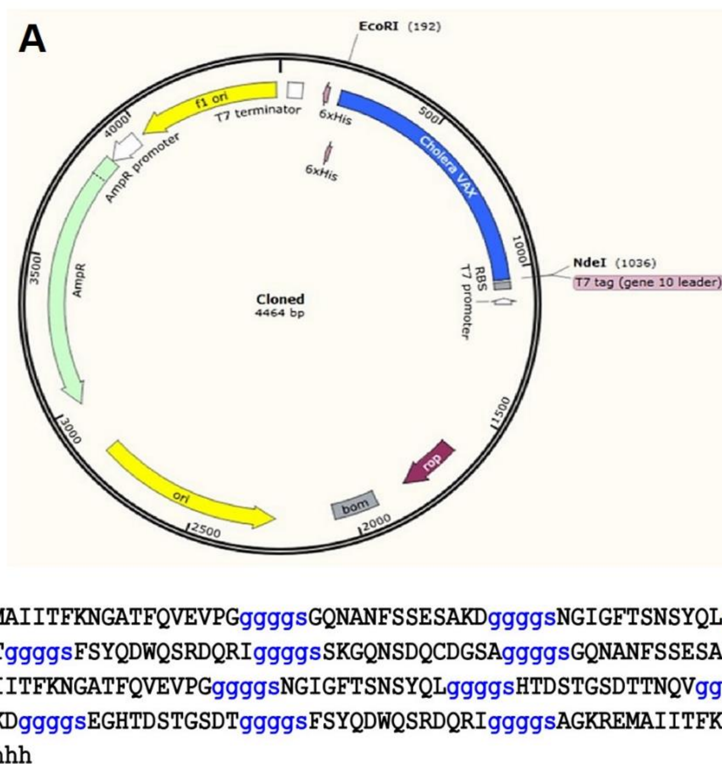


Figure 1. Simulated DNA encoding polypeptide in pET23a(+) expression plasmid; *in silico* cloning was done through NdeI and EcoRI restriction sites of the pET system (A). Amino acid sequence of designed polypeptide (B).

polypeptide nucleotide sequence in pET23a(+) plasmid was simulated using the SnapGene offline software. Figure 1 shows the simulatation of the restriction cloning into pET23a(+) plasmid (Figure 1A) and the amino acid sequence of the designed polypeptide (Figure 1B). To design a multi-epitope polypeptide, various factors such as surface accessibility, antigenicity, hydrophilicity, and flexibility were considered (Figure 2). The antigenicity of the designed polypeptide was calculated about 2.0826, which indicates that the designed polypeptide has a suitable antigenicity.

Validation and Evaluation of Physicochemical Properties of the Designed Polypeptide

Validation of the designed polypeptide has been performed by using different servers which results are presented in the following. Regarding the results of the Protparam server and Pepcalc database, the designed polypeptide contained 277 amino acids, and Gly and Ala are the most constitutive amino acids in the designed polypeptide. The instability index (II) is computed to be 39.50 which classifies the protein as stable. The estimated half-life was 30 hours in mammalian reticulocytes (*in vitro*), >20 hours in yeast (*in vivo*), and >10 hours in Escherichia coli (*in vivo*). The Grand average of hydropathicity (GRAVY) of the designed polypeptide was -0.820. Also, the estimated solubility of the designed polypeptide was good water solubility (Figure 3).

GOR IV secondary structure prediction method for the secondary structure of the designed polypeptide contains; alpha-helix (6.50%), random coil (70.76%), and extended strand (22.74%) (Figure 4). As mentioned before, the I-TASSER server was used for the prediction of the 3D structure of designed polypeptide peptides that resulted in five tertiary models of the input data based on their values (C-score = -2.13, Estimated TM-score = 0.46 ± 0.15, Estimated

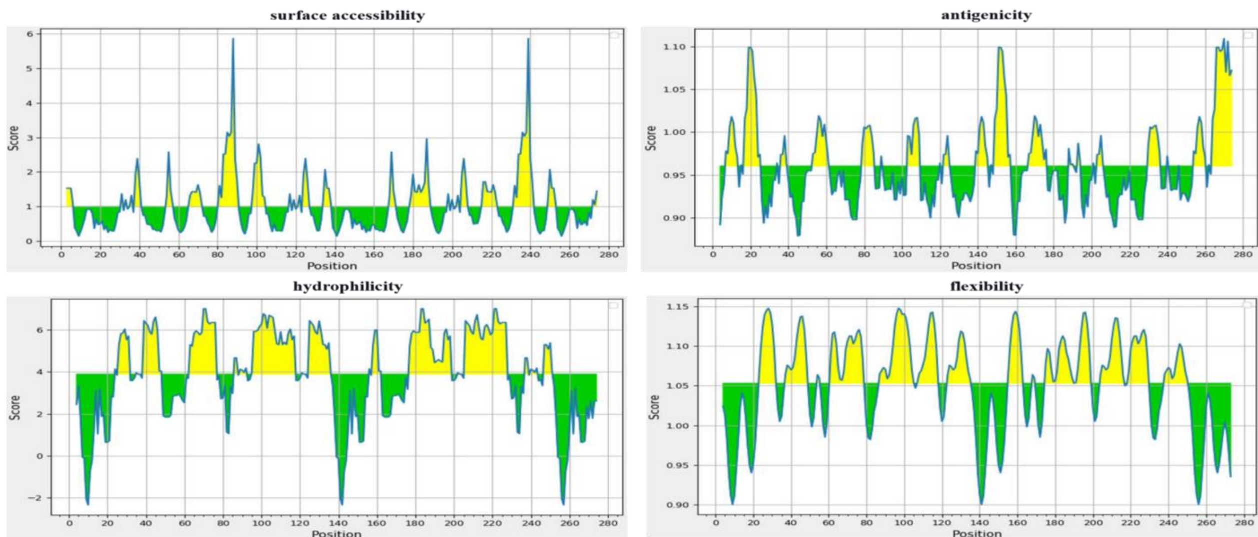
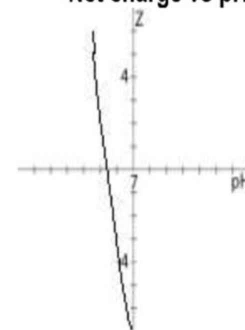


Figure 2. Analysis of the Designed Polypeptide Features such as Surface Accessibility, Antigenicity, Hydrophilicity, and Flexibility.

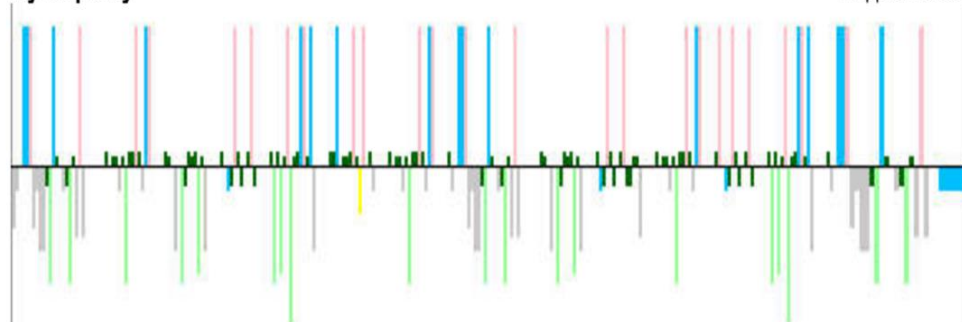
Physiochemical properties

Number of residues:	277	
Molecular weight:	27075.93 g/mol	notes on MW
Extinction coefficient:	16500 $M^{-1}cm^{-1}$	notes on Ext. Coefficient
Iso-electric point:	pH 5.43	notes on pI
Net charge at pH 7:	-7.2	notes on net charge
Estimated solubility:	Good water solubility.	notes on solubility

Net charge vs pH



Hydropathy Hopp & Woods



Top is hydrophilic
Bottom is hydrophobic
Color codes: Acidic Aromatic Basic Aliphatic Polar Cysteine

Figure 3. Secondary Structure Analysis of the Designed Polypeptide Using the Gor4 Method. The most constative secondary structure of the protein is in the random coil form. Also, 6.50% and 22.74% of protein contain alpha helix and extended strand, respectively.

RMSD = 10.9 ± 4.6Å). While the 3D structure of the designed polypeptide was predicted, the GalaxyWeb server was used to refine the selected model of I-TASSER. On this occasion, ten refined models for the input structure were suggested by the server. Subsequently, the proposed models were compared based on several factors such as the RMSD, the MolProbity and clash score, poor parameters, Rama favored, and galaxy energy in a table. Finally, model 1 was

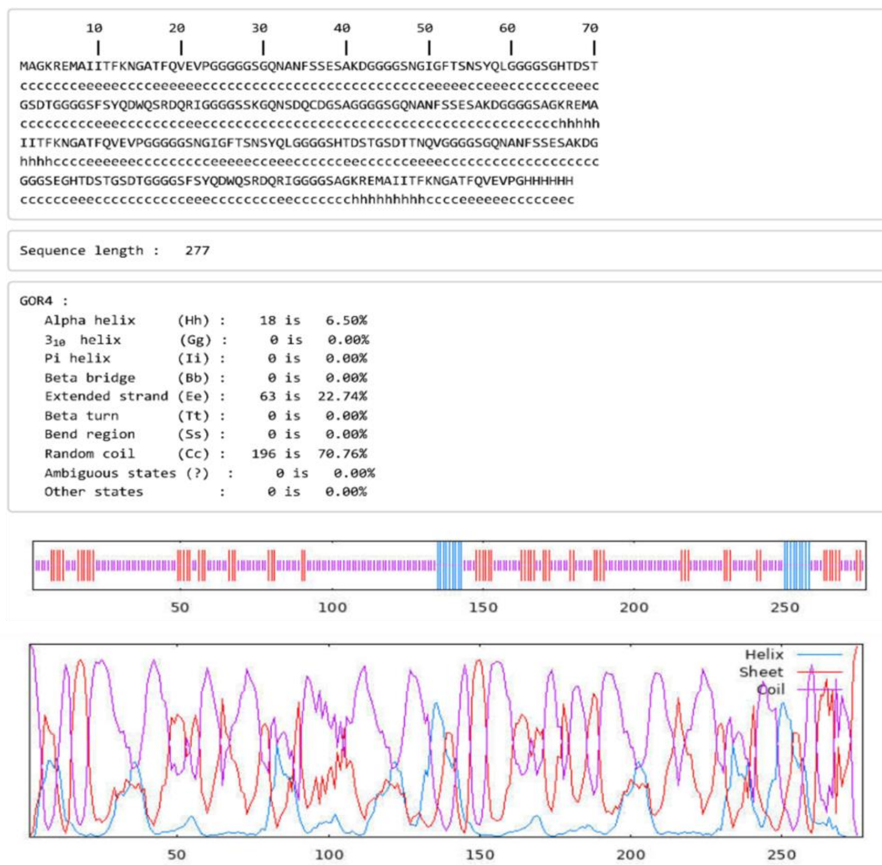
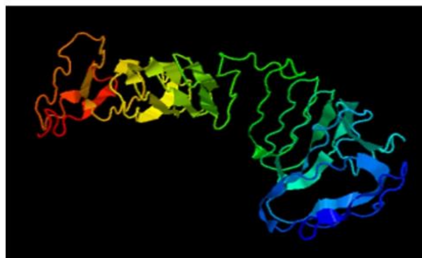
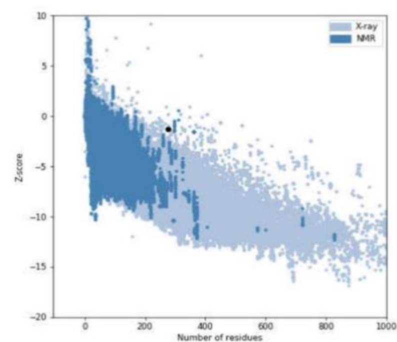


Figure 4. Analysis of the Physicochemical Properties of the Designed Polypeptide Using the Pepcalc Database.

A



B



C

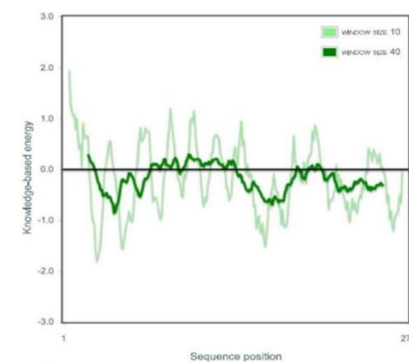


Figure 5. The best predicted tertiary structure of designed polypeptide created by I-TASSER (A); C-score = -2.13, Estimated TM-score = 0.46±0.15, Estimated RMSD = 10.9±4.6E. Analysis of the designed polypeptide using the proSA web; Overall model quality (B), Local model quality (C).

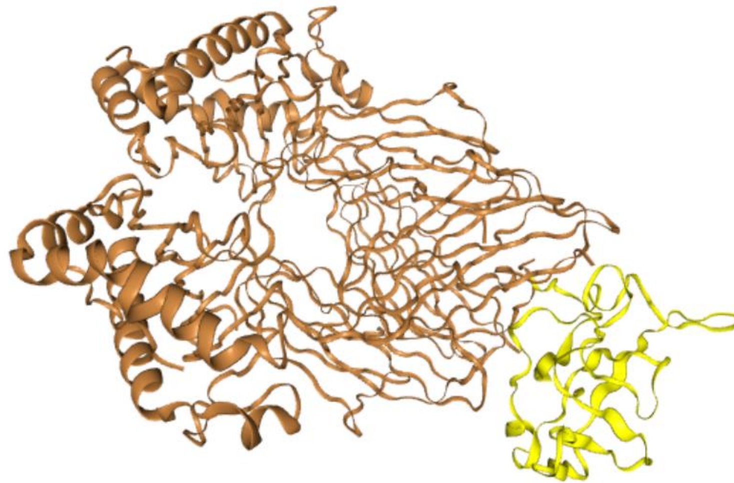


Figure 6. The best docking mode for the designed polypeptide (right) and MHC II complex (left), predicted by HDOCK server. Docking score = -218.97, Confidence score = 0.7989, Ligand rmsd (A°) = 112.34.

selected after considering the suggested models (Figure 5A). The results showed that in the selected model, 80.8% of amino acids are located in the most desirable regions. The clash score and the galaxy energy for the selected model were 6.0 and -3161.78, respectively. In addition, depict root mean square deviation (RMSD) for the selected model 1, reported 5.299 which was the highest amount among the refined models. Furthermore, the ranking of the suggested refined models in this server was based on the RMSD score and Rama favored regions which directly related to the quality of the predicted model. Then, the Ramachandran plot was applied to validate the quality and geometric features of the selected model. Accordingly, about 80.8% of the amino acids were located in the most favored regions. Finally, the proSA analysis gave a Z-score of −1.28 for the selected model (Figure 5).

Molecular Docking

The best docking scores for the complexes were -209.27 and -218.97 for the designed polypeptide- MHC I and - MHC II complexes, respectively. The results of this part confirmed that the designed polypeptide has a relatively better tendency to bind with MHC II molecules. Figure 6 shows the best docking mode with the highest confidence score for the designed polypeptide–MHC II complex by the HDOCK server.

Discussion

The reverse vaccinology (RV) strategy has a range of applications and has been reported in a variety of vaccine design investigations against infectious diseases. However, it was first applied to the Meningococcus B serotype.[4] Multi-epitope peptide-based vaccines are a new generation of vaccines that can activate humoral and cellular immune responses, at the same time.[34] Dar and colleagues have designed a multi-epitope vaccine against Klebsiella pneumoniae using the RV approach. They have selected four immunogen proteins among the 222 pan genomic sequences for the bacteria. Moreover, they have predicted nine B cell-derived T cell epitopes to construct their vaccine in order to reduce the length of their final construct.[35] In another study, Khan and colleagues have developed an epitope-based vaccine against the Ebola virus due to prevent the spread of the related disease.[36] They have utilized nine amino acids to predict the most potential B and T cell epitopes of this virus which could interact with 12 HLAs with high population coverage of up to 80.99%. Zeinoddini and colleagues designed a new multi-epitope peptide-based vaccine candidate against Q fever. In this investigation a molecular docking study was performed to measure the affinity of binding the proposed vaccine to MHC I and II molecules and found out the designed epitope-based protein can be used as a potential vaccine to provide protection against Coxiella burnetii.[37] The RV method has also been implemented in the current pandemic of the Covid-19, whereas, several papers were published by researchers in this field. Chen and coworkers designed an epitope vaccine against the pandemic's causative agent.[38] In that study, they predicted linear and discontinuous B and T cell epitopes of the spike protein predicted by using ABSpred and Discotope-2.0 and the IEDB server, respectively. In another study related to Covid-19 infection spread, Samimi et al designed a peptide-based multi-epitope vaccine against coronavirus2 highly infectious disease.[39] This research group chose various genes of the coronavirus in order to develop their vaccine, and they finally reported that their designed vaccine has a reliable interaction with MHC molecules and Toll-like receptors 3 and 4 (TLR).

This work aimed at designing and development of a new multi-epitope polypeptide candidate for cholera infection

using various bioinformatics servers and the reverse vaccinology approach. Due to the fact that an appropriate epitope should have a proper antigenicity without allergenic and toxic properties, these characteristics were identified and investigated through this study. As science develops in bioinformatics, various databases and servers have been created to raise the rate of algorithm performance and prediction methods. For instance, a variety of databases are accessible for the design and validation of different types of vaccines against pathogens and cancers.[40,41] Therefore, in the present work, we have applied chromosome I of the *V. cholerae O1* for the epitope mapping process. The chance of success in the vaccine development process could be increased if numerous pathogen-related proteins were considered for the epitope mapping process. The other vital point here is the population coverage, which is a crucial issue for the development of a universal vaccine. To this end, in this study, we tried to choose the epitopes that covered most of the MHC I and II alleles in different populations. The molecular docking study was performed to validate the binding energy between the designed polypeptide and MHC I and MHC II complexes, separately.

Conclusion

This investigation aimed to design a multi-epitope polypeptide candidate against *V. cholerae O1* using immunoinformatic based on a reverse vaccinology approach. Various methods and algorithms were utilized to predict reliable, immunogen and non-allergen epitopes. The molecular docking results have also confirmed the interaction between the proposed designed polypeptide and MHC I and II molecules.

Authors' Contributions

ASN: writing and original draft preparation; ARS: project administration, investigation, methodology, data curation, manuscript editing; MZ: methodology, validation; RM: investigation, data curation, manuscript editing.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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