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Designing of Chimeric Vaccine against *Canine Distemper* Virus Targeting Hemaglutanin Protein

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Abstract: The canine distemper virus is highly contagious and affects dogs' respiratory systems. The virus belongs to the paramyxoviridae family and order Mononegavirales. This class of viruses contains a negative-strand RNA. This virus also affects raccoons, foxes, and other animals. The current study aims to design a vaccine against the virus employing reverse vaccinology. The target candidate for the vaccine design is a surface protein called Hemagglutinin. Viral hemagglutinin protein sequences were retrieved from the Uniprot database, and conserved regions were identified. Possible B-cell epitope regions were identified using the ABCpred server. These epitopes were analyzed for allergenic and antigenic properties using the Allergen FP server and VaxiJen v2.0 server. The epitopes, which were antigenic and non-allergenic, were screened for T cell epitopes using NetMHC and NetMHC2 servers. The toxicity of the selected peptides was evaluated using the Toxinpred server. The epitopes were further screened for transmembrane helices and signal peptides employing TMHMM v. 2.0 and SignalP 4.1 servers, respectively. The epitopes were then checked for the parameters using the ProtParam tool. Finally, the solubility of the epitopes was determined using the SOLPro server. Using the selected epitopes, a chimeric vaccine construct was constructed with the peptides by linking the peptides with the GPGPG linker to the cholera toxin subunit B. The chimeric vaccine was modeled using the Robetta server, and codon optimization of the construct was performed using the JCAT tool.

Keywords: canine distemper virus; vaccine; morbillivirus; immunoinformatics; epitope.

1. Introduction

Canine distemper virus, abbreviated as CDV is known to cause a highly contagious disease that affects a broad range of animals. CDV affects Canidae, which includes domestic dog, coyote, jackal, wolf, dingo, and fox; Mustelidae, which includes animals like minks, skunks, ferrets, otter, weasels, and badgers; Procyonidae family, which encompasses raccoons, coatis, and pandas; Ursidae comprising bears; Viverridae that includes genets, civets, and linsangs and Hyaenidae, Felidae and Ailuridae family of animals including hyenas, lions, and tigers, lesser and giant pandas, respectively. It also affects humans, other mammals, and aquatic animals [1-3]. CDV is a single-stranded negative-sense RNA that belongs to the family Paramyxoviridae and the genus Morbillivirus [1,3-7]. Despite several control measures, CDV severely affects dogs [1,4]. The infected hosts manifest various systemic and neurological clinical symptoms [2].

CDV is a highly contagious pathogen that is majorly transmitted through direct contact with an infected animal, bodily secretion of infected animals, or via contaminated aerosols [4].

CDV is known to affect several vital body parts, including the spinal cord, brain, respiratory and gastrointestinal tract. It exhibits a wide range of symptoms in the infected hosts. Clinically recorded symptoms include fever, serous oculo-nasal discharges, anorexia, bronchitis, conjunctivitis, pneumonia, gastroenteritis, coughing, loss of appetite, vomiting, diarrhea, nervous signs, and skin lesions such as pustular or vesicular dermatitis and hyperkeratosis [2,8]. Within 20 to 24 hours post-infection, the virus multiplies within macrophages, B cells, and T cells circulating in the blood. After the replication, viral particles enter the lymphatic system and the bronchial lymph nodes [9,10]. Immunosuppression is triggered during the initial viral replication process when CDV enters the lymphatic system [11,12]. During the viral replication inside the lymphatic system, the host exhibits hyperthermia and leukopenia due to low lymphoid cells [13]. CDV then spreads through hematogenous pathways or cerebrospinal fluid (CSF) and enters the epithelial tissues and central nervous system (CNS). Neurological symptoms remain unpredictable and owe to infected animals' mortality [10].

The RNA genome of CDV translates to six structural proteins - nucleocapsid (N), matrix protein (M), fusion protein (F), hemagglutinin glycoprotein (H), phosphoprotein (P), and Polymerase (L), and two non-structural C and V proteins [14]. The H proteins are the keystone protein for CDV as they have a role in the host animals' immune interaction [15,16]. The first step of infection involves CDV attaching its Hemagglutinin protein to the host cell via receptors on the surface of the host's cell [17]. Vaccines developed to protect against CDV are usually the attenuated form of the virus [18,19].

In general, when the pathogen enters the host, the host's immune system recognizes them in response to the invading pathogen. The immune cells recognize and respond to the selective regions on the pathogen called antigens or epitopes over an entire organism. The immune response provides the host with protection and the desired immunity. This established the concept of designing epitope-derived vaccines. This method uses selective antigens or epitopes derived from the genome present in the pathogen's immunome. This helps to elicit the required immune response, and therefore, it confers protection against the same pathogen during future invasions. Identifying these epitope/antigenic regions from large protein pools is a herculean task. But, with the availability of state-of-art computational tools and software, it has become very convenient to achieve the same. Immunoinformatics tools are important in identifying and analyzing antigens/epitopes, which can elicit an immune response. Another added advantage of using these computational tools is effectively reducing the time and cost of designing such vaccines. [20,21]. We have attempted to design a chimeric epitope vaccine against CDV targeting the Hemagglutinin protein in the current study using an *in-silico* Immunoinformatics approach.

2. Materials and Methods

In-silico vaccine design development involves a series of steps, from selecting the target protein to the complete construction of the vaccine. The process utilizes cost-effective Immunoinformatics tools and reduces the risk of intervention failure [22].

The epitopes were selected based on the following criteria: (1) 10-mer peptides should be highly conserved, which can be found by comparing the sequence with the results of MSA, (2) Antigenic, (3) Non-allergen, (4) Non-toxin, (5) Soluble, (6) Stable, (7) Absence of signal peptides and TMHMM and (8) Potential to bind with the maximum number of MHC-I and MHC-II alleles having a cut-off value of IC50 between 0.01 and 1000 nM.

2.1. Protein retrieval and identification of conserved regions.

The first step of the vaccine design is to identify the surface proteins present in the virus. Hemagglutinin glycoprotein, a surface protein in CDV, was selected for the vaccine design. The protein sequence was retrieved in FASTA format from the UniProt database [23]. To detect sequence homologs with *Homo sapiens*, BLASTp was done [24]. The Clustal Omega server performed multiple sequence alignments of the amino acid sequence of the sequences [25].

2.2. B cell epitope.

ABCPred server - Artificial Neural network-based B-cell epitope prediction (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html) was employed to mine the B-cell epitopes from the sequences. The parameters for sequence selection are sequence length of an epitope should be greater than 10, and the threshold was fixed to 0.51. Based on the results, nine epitopes were selected [26].

2.3. Antigenicity, allergenicity, and toxicity assessment for selected epitopes.

These nine epitopes were analyzed to check their properties. Antigenicity of the selected epitopes was predicted using the VaxiJen server. The threshold was set to 0.4 [27]. Further, the allergenicity of the selected peptides was checked using the Allergen FP server [28]. Among the nine epitopes, only one epitope was antigenic and non-allergenic. This was further subjected to toxicity analysis using. Toxinpred server. It is an *In-silico*-based tool that predicts the toxicity of the epitopes and develops models based on motif information to identify the frequency of amino acids, which can be considered if it could cause toxic effects [29].

2.4. Prediction of IFN epitopes and physicochemical parameters.

The screened epitope was assessed whether it was IFN-γ inducing using the IFN epitope server. The Interferon-gamma (IFN-γ) can interfere with the viral replication, activating the T-cells, Natural Killer cells (NK), and other macrophages and passing signals to nearby cells to produce anti-viral proteins such that it can degrade the viral antigens [30]. Evaluation of various parameters, including physical and chemical properties of the peptides, was checked using the ProtParam tool [31]. The presence of possible signal peptides was predicted using the Signal P 4.1 server [32]. The THMM server v.2.0 was used to predict the presence of any transmembrane helices in the selected epitope [33]. SOLpro server was used to predict the solubility of the peptides upon gene expression in *Escherichia coli* [34].

2.5. Prediction of T cell epitopes.

The T-cell epitopes are the peptide fragments of 8–11 amino acids and can elicit specific immune responses. T-cell epitopes derived from the B-cells can elicit both humoral and cellular immunity. The server NetMHC4.0 was used to predict the MHC-I binding epitopes. Eighty-one human leukocyte antigen (HLA) alleles (HLA-A, B, C, and E) and six murine alleles (H-2) were evaluated. Predictions were made for ten-mers epitopes with a threshold setup of 0.5% for strong and 2% for weak binders. The MHC-II binding epitopes were predicted by the Net MHCII 2.3 server. Predictions were obtained for 25 - HLA-DR alleles, 20 - HLA-DQ, 9 - HLA-

DP, and 7 - mouse H2 class II alleles using a threshold of -99.9 for the strong binder of 2%, and a threshold for the weak binder of 10% [35-36].

2.6. Design of the chimeric vaccine.

The cholera enterotoxin subunit B amino acid sequence was retrieved from the Uniprot database (Uniprot entry: P01556) and added as an adjuvant to which the selected five epitopes were linked together at the C-terminal end with the help of Gly-Pro-Gly-Pro-Gly (GPGPG) linkers [37]. The modeling of the construct was performed using the Robetta server, which employs a deep learning-based modeling method [38]. The all-atom contact analysis of the MolProbity server validated the model, which shows high-accuracy Ramachandran and rotamer distributions. The Ramachandran plot is based on phi and psi dihedral angles which can verify the backbone conformation of the protein structures by analyzing each residue and classifying them into allowed, favorable, and outlier regions [39].

2.7. Evaluation of the physicochemical properties of the constructed vaccine.

VaxiJen server was used to predict antigenicity with the threshold value of 0.4, and AllergenFP predicted allergenicity. The SOLpro server evaluated the solubility of the protein, and ToxinPred predicted the toxicity of the vaccine construct. The TMHMM server can predict the transmembrane regions. ProtParam allowed the computation of various physical and chemical parameters. The instability index value can predict the stability of the protein. The instability index value is <40 for stable protein and >40 for unstable protein.

2.8. Codon optimization.

To express the foreign genes into a host, the Java Codon Adaptation Tool (JCAT) plays a major role in making the codon usage adapt to most sequenced prokaryotes and eukaryotes (https://www.jcat.de/). The occurrence of unadapted codons can lead to a minor expression rate, whereas adapting codons can lead to successful protein expression in the host with a higher rate [40].

3. Results and Discussion

Traditionally, vaccines are prepared by inactivating or attenuating the infectious pathogen, which may induce a host immunological response. But the issue with these vaccines is that they may be allergenic or toxic to some. So, recombinant vaccines like the subunit and conjugates vaccines were developed. This is designed to target a particular protein or toxin. But this method also suffers serious drawbacks. Bioinformatics tools are employed to develop multi-epitope vaccines by employing a reverse vaccinology strategy. This ensures that the vaccines that are designed are antigenic but not allergenic/toxic. Using such in-silico tools are also advantageous as they reduce the time and expense of designing such a vaccine.

In-silico Vaccine design employs the identification of epitopes within the target protein to construct a chimeric vaccine. Epitopes are regions in the protein that are highly antigenic—, which are also known as epitopes. The chimeric vaccines are constructed with suitable adjuvants to enhance the immunogenicity of the vaccine. Usually, the targets chosen for the vaccine design could be from the pathogen's core proteome and non-redundant. In general, extracellular proteins, i.e., surface proteins, are chosen for the study and are subjected to various filters to select a potential epitope. Proteins are examined for allergenicity, antigenicity,

toxicity, the presence of transmembrane helices, and physicochemical properties like stability, solubility, etc., to screen effective and feasible vaccine targets [41-45]. The complete workflow of the design of the vaccine construct is shown in Figure 1.

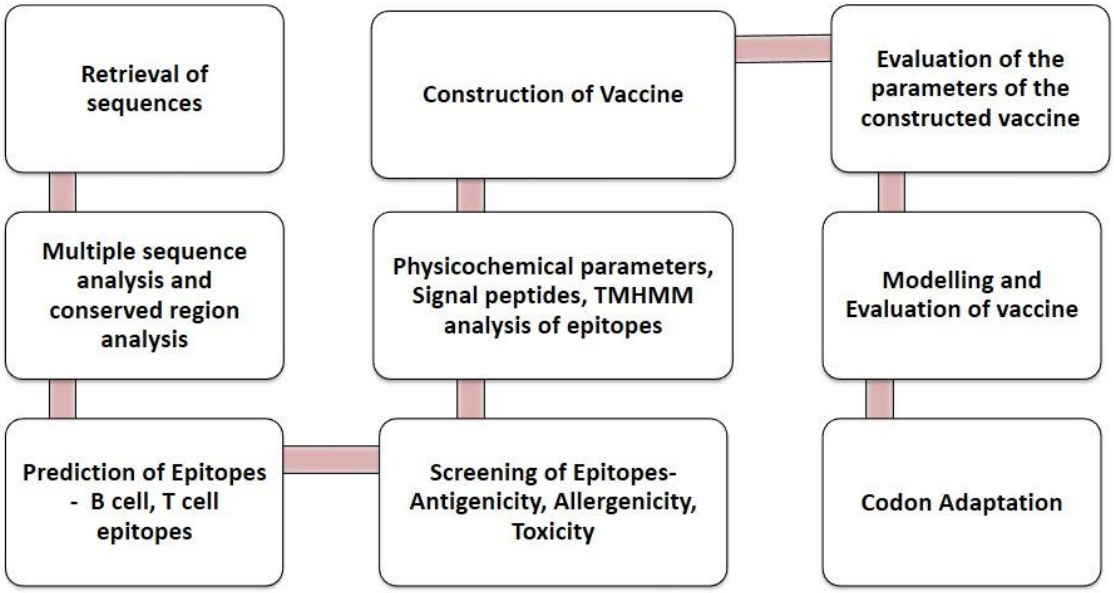

Figure 1. Flowchart of selecting potential epitopes for vaccine construction.

3.1. Retrieval of proteins & Identification of conserved regions.

The CDV contains surface proteins like Hemagglutinin, fusion glycoprotein, nucleoprotein, and other matrix proteins. Among these, the surface protein hemagglutinin was selected owing to the stability of the protein. The hemagglutinin glycoprotein of the canine distemper virus was searched in the Uniprot database. Around 857 hemagglutinin glycoprotein hits were obtained. From the sequences, gold star sequences were downloaded in FASTA format and analyzed further. The sequences were subjected to BLASTP against canine organisms and confirmed to have less than 40% similarity index compared to any known canine protein. The homology of the protein sequences was checked with Homo sapiens using BLASTP to find the non-homologous sequences. The multiple sequence alignment of the protein sequences laid out the presence of amino acid conserved residues performed by the Clustal Omega tool. It was found that the eleven protein sequences were 95% similar to having a single, fully conserved residue represented as (*), which was ideal for the multivalent peptide vaccine design, which can confer long-lasting immunity. These protein sequences could act as antigens that can be regarded as potent epitopes based on stimulating an immunogenic protective response. Around 9 conserved regions were identified as having identical residues listed below in Table 1. These residues can act as an antigen and, hence, as an epitope for inducing immunogenic response, basic essentiality for a vaccine construct.

Table 1. List of Conserved regions.

S. No	Conserved Region
1	GAFYKDNARAN
2	DVLTPLFKIIGD
3	RLPQKLNEIKQFILQ
4	KTNFFNPNREFDFRDLHWCINPPS
5	RCSGATTSVG
6	IRVFEIGFIKRWLN
7	VCTIAVGEL
8	LNISFTYGPVIL

S. No	Conserved Region
9	FRLTTKGRPD

3.2. Prediction of linear B-cell epitopes.

All the conserved protein sequences were taken forward for Linear B-cell prediction using the ABC Pred server to screen out the possible epitope regions arising from the protein sequences. In this regard, the possible epitope regions were determined for the hemagglutinin protein. A threshold value (>0.85) was set. This threshold value was decided upon to achieve more sensitivity between 95.5% and 99.5% for the epitope prediction.

3.3. Antigenicity, allergenicity, and toxicity assessment for the selected epitope.

Antigenicity is an important factor which constructing chimeric vaccines as it determines the success of such vaccines. In order for the designed vaccine to elicit an immune response, it needs to be immunogenic and antigenic. So, the antigenic and non-allergenic nature of peptides is crucial in selecting the peptides for further assessment. The predicted B cell epitopes were checked for antigenicity, allergenicity, and toxin properties through VaxiGen 2.0, Allergen FP v1.0, and Toxin Pred, respectively. Peptides with a score > 0.4 were considered antigenic and were taken up for further study. A total of six out of nine B-cell epitopes were screened as potential antigens. The Allergenicity results showed that there were 5 allergens, of which 1 were non-allergen epitopes with a Tanimoto similarity value index above 0.60. ToxinPred tool works on Support Vector Machine (SVM). This predicts peptide toxicity along with the physicochemical properties of peptides. The 1 potential epitope - EFDFRDLHWC, was screened, which was non-toxic.

3.4. Prediction of transmembrane analysis and signal peptides.

The THMM server results show that all the potential epitopes do not have transmembrane helices and might play a crucial role in showing antigenicity and accessibility to the immune system. The absence of signal peptide cleavage sites predicted by the Signal P server illustrates that no signaling proteins are present in the potential epitopes.

3.5. Physiochemical properties of the selected epitope.

The Protein parameters were analyzed using the ExPasy Protparam tool. The total amino acid residues were 10, and the molecular weight was 1367.5 Daltons, whose molecular formula is $C_{63}H_{82}N_{16}O_{17}S_1$. The pI theoretical value was 4.54. One positive residue and three negative residues were found with a half-life period *in vitro* of an hour in mammalian reticulocytes and 30 mins yeast and greater than ten hours in *Escherichia coli* in in vivo models. The epitope was non-toxic and stable.

3.6. T cell epitope prediction.

The selected epitope was assessed for its potency to act as a T cell epitope. The epitope was analyzed using NetMHC 4.0 and NetMHC 2.3 servers for MHCs Class 1 and 2, respectively. The HLA super representative (HLA D) induced 12 alleles, HLA A, B, C, and E were inducing 20,20,10,1 alleles, respectively. The mouse H2 units induced 5 alleles, and the DR, DP, DQ, and H2 subunits induced 25, 8, 17 and 7 alleles, respectively. Hence the test

results had more favorable alleles, as the epitope induced a considerable number of alleles in both the classes with the high-affinity score.

3.7. Vaccine construction and modeling.

The selected epitope "EFDFRDLHWC" sequence was subjected for vaccine construction using Cholera Enterotoxin subunit B amino acid sequence (125 amino acids in length) as adjuvant and GPGPG (Glycine-Proline-Glycine-Proline-Glycine) as linker at the C-terminal. Linkers play an important role in the structural and functional aspects of the designed chimeric protein. It also helps in representing the individual epitopes as an entire structure in the vaccine. Flexible linkers used in constructing the vaccine improve the structure's flexibility and protein stability and enhance the designed vaccine's biological activity [37]. The constructed vaccine model sequence is as below,

MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIYTLNDKIFSYTESLAGKREM AIITFKNGAIFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHA IAAISMAN GPGPG EFDFRDLHWC GPGPG

Three-dimensional modeling of the vaccine construct gives a complete idea of the key structural elements in the designed protein. It helps in understanding the interaction of the ligand with the protein and also aids in the molecular dynamics of the protein. The Robetta server was used to generate models for the vaccine construct, and the best model was chosen by validating the model using the Ramachandran plot. The Robetta server-generated models for the multi-epitope vaccine construct and the best model were selected based on the analysis of generated Ramachandran plots. The best vaccine construct model shown by the Ramachandran plot displays 95.9% of all residues in the 98% favored regions and 98.4% of all residues in the allowed regions (>99.8%). The three-dimensional model of the vaccine construct is shown in Figure 2.

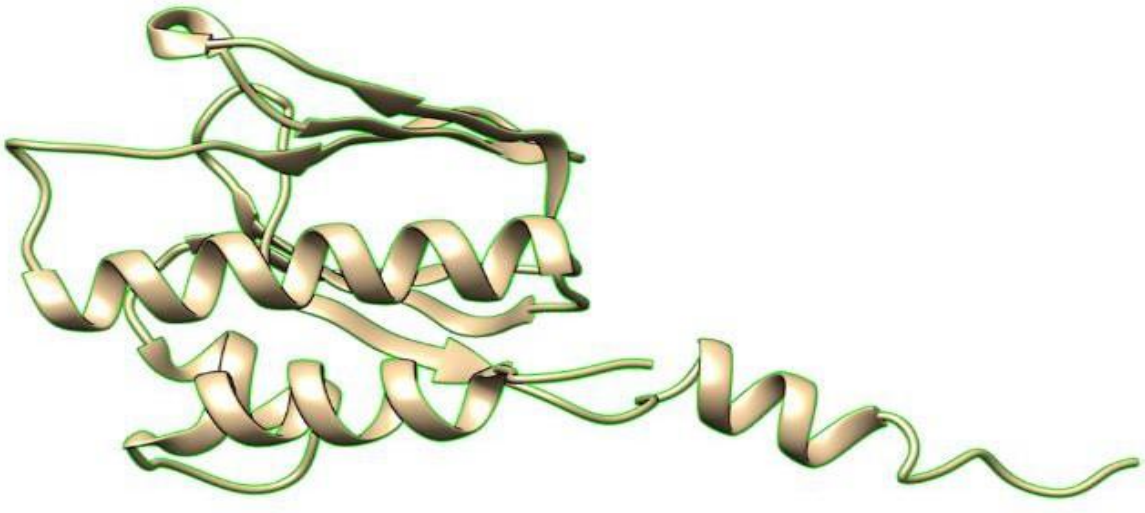

Figure2. Three-dimensional model of the chimeric vaccine construct.

3.8. Physiochemical analysis after vaccine construction.

The designed vaccine construct was evaluated for its properties, and the data are tabulated in Table 2.

Table 2. Physiochemical properties of the constructed vaccine.

S. No	Properties	Score or Results
1	Antigenicity	Antigen (Score = 0.6146)

2	Allergenicity		Non-Allergen (0.61)
3	Protein Properties	Amino acid count	144
		Molecular weight	16037.44 Daltons
		Molecular Formula	$C_{63}H_{82}N_{16}O_{17}S_1$
		pI (Theoretical)	7.74
		(-)ve charged residues	14
		(+)ve charged residues	15
		Half-life	(Mammalian reticulocytes, in vitro), 30 hours (Yeast, in vivo), 20 hours (Escherichia coli, in vivo) >10 hours
		Stability	Stable
4	Toxicity		Non-Toxic (Score = -0.78)
5	Solubility		Soluble (Score = 0.610123)
6	Signal Peptide		Nil
7	Transmembrane proteins		0

3.9. Codon Optimization.

The Codon optimization was done with the Jcat server's help to perform possible codon sequence adaptions in pre-existing common prokaryotic and eukaryotic organisms sequenced. For this study, *E. coli* strain k12 was chosen to identify codon adaptations. The essential properties of gene sequence should lie under the given criteria to achieve high expression levels in the host. The best CAI (Codon Adaptation Index) value is found to be 1.0, while a score greater than 0.8 is regarded as a good score. The total GC content range should lie from 30 to 70%. The CAI value was 1.0, and the GC content of the improved sequence belonging to *E. coli* host strain K12 was 56.66, indicating that high expression occurs in *E. coli* as a host. The improved DNA sequence is as follows:

ATGATCAAACTGAAATTCGGTGTTTTCTTCACCGTTCTGCTGTCTTCTGC 50

TTACGCTCACGGTACCCCGCAGAACATCACCGACCTGTGCGCTGAATACC 100

ACAACACCCAGATCTACACCCTGAACGACAAAATCTTCTCTTACACCGAA 150

TCTCTGGCTGGTAAACGTGAAATGGCTATCATCACCTTCAAAAACGGTGC 200

TATCTTCCAGGTTGAAGTTCCGGGTTCTCAGCACATCGACTCTCAGAAAA 250

AAGCTATCGAACGTATGAAAGACACCCTGCGTATCGCTTACCTGACCGAA 300

GCTAAAGTTGAAAAACTGTGCGTTTGGAACAACAAAACCCCGCACGCTAT 350

CGCTGCTATCTCTATGGCTAACGGTCCGGGTCCGGGTGAATTCGACTTCC 400

GTGACCTGCACTGGTGCGGTCCGGGTCCGGGT

4. Conclusions

The canine distemper viruses are one of the viruses affecting the canine organisms, and hence a vaccine was designed for the same by analyzing the surface protein hemagglutinin; with the studies carried out, a suitable epitope for the construction of the vaccine has been identified. The epitope was stable, antigenic, non-allergenic, soluble, and non-toxic, all essential parameters for vaccine construction. After preliminary studies, the epitope was linked with cholera toxin subunit B, and a 3D model was generated. The constructed vaccine and model were also tested for their stability, codon optimization, and other protein parameters.

The results of the conducted in silico studies strongly support that the modeled vaccine is potential for further in-vivo studies.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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