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ORIGINAL RESEARCH

Immunoinformatics driven construction of multi-epitope vaccine candidate against *Ascaris lumbricoides* using its entire immunogenic epitopes

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ABSTRACT

Objective: *Ascaris lumbricoides* infects 80 million people per year, causing malnutrition, stunted growth of children etc., but there is no vaccine available against it. We aimed to design a multimeric-subunit vaccine using comprehensive immunoinformatic approach.

Research design and Methods: The T and B cell epitopes were shortlisted on antigenicity, allergenicity, and toxicity from proteome data and joined with appropriate linkers. The physical characteristics of vaccine candidate was calculated and docking/molecular dynamic simulation performed to validate its robustness. The multimeric protein was codon optimized and in-silico cloned in pET28b.

Results: From the 23,604 proteins of Ascaris, we filtered based on epitope prediction, localization, antigenicity, and allergenicity. Prepared a vaccine of 534 amino acid long, 56.31 kD weight and pI 4.52. Physiochemical features showed it is soluble, highly antigenic and non-allergenic. Its tertiary structure was forecasted, certified, and refined. The immunoinformatic simulation studies showed it to be potent T and B cell stimulator.

Conclusions: We identified highly antigenic peptides of Ascaris from its proteome with good potential to induce innate as well as humoral immune response. These peptides were used to design a chimeric vaccine against Ascariasis infection, which can be used for prophylactic purpose but needs experimental and clinical validation.

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Ascaris lumbricoides; infection; parasite; vaccine; immunoinformatics; helminths

1. Introduction

Ascaris lumbricoides is a soil-transmitted helminth, (STH) which causes small intestinal infection in humans. It is one of the most common STHs in developing countries [1,2] and endemic in tropical and temperate parts of the world. Poor hygiene and sanitary conditions, lack of awareness among residents about the disease, and conducive environmental conditions help parasite to maintain its life cycle. Although *A. lumbricoides* has been eradicated from most of the developed countries but it is estimated that 0.81–1.20 billion individuals in the world are harboring this parasite in their gastrointestinal tract in endemic areas [3]. According to World Health Organization (WHO) data published in 2018, in India 429,557,443 children require preventive chemotherapy for STH but only 195,062,594 children received it [4,5].

Ascaris resides in jejunum of human small intestine but it has ability to migrate to other body parts such as lungs, liver, gallbladder, and pancreas [6]. Adult worms reside in intestinal lumen of human host and has the potential to produce 200,000 eggs daily which are defecated/excreted along with feces. These eggs can survive under extreme environmental conditions. The fertilized eggs are transformed into embryonated eggs which develop into larvae. When the climate is conducive, like moderate warmth (25–30°C temperature), high moisture content and adequate supply of oxygen, these fertilized eggs molt and embryonate in a span of 15–35 days to achieve infective stage. The infective eggs carry a 2nd stage larva, coiled within its own eggshell. The infective eggs reach human host due to consumption of contaminated food having embryonated eggs. In gastrointestinal tract they get transformed into larvae and circulate to the lungs and other organs. From the lungs, larvae are coughed out or swallowed sometimes and thus they re-enter the gastrointestinal tract. Maturation process proceeds in the small intestines [7,8]. The Ascariasis symptoms varies from asymptomatic phase to severe symptomatic phase with warning signs of infection, these signs increase with increasing parasitic burden, which leads to intestinal obstruction, loss of appetite, intestinal bleeding, and malnutrition in children [9].

At present, there is no vaccine available for ascariasis. The pan-anthelminthic vaccine was developed by the Sabin Vaccine Institute against STH including Ascariasis, Trichuriasis and Hookworm infections. For this vaccine, proteins from other than *A. lumbricoides* species were used because it was observed that different Ascaris species share similar proteome data. For example *A. suum* that infects pigs shares specifically lung stage protein profile with *A. lumbricoides*

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Supplemental data for this article can be accessed here.

[10,11]. In a recent study, crude homogenate of adult *A. suum* was used to vaccinate BALB/c mice, which induced protective IgG response but there was no difference in parasite load between control and vaccinated mice [12]. Similarly in another attempt to make a vaccine, a chimeric vaccine had shown promise by inducing 74% protection in a mice study [13]. Passive immunity is another way to generate immunity against any infection and provide better results, in the case of ascariasis infection, IgG antibodies from diseased mice to naïve mice reduced the parasite load by 65% [10]. The *A. suum* enolase is highly conserved between different nematodes and it is reported that it reduced the 61.13% parasite burden in mice and can act as a better vaccine candidate [14]. But a detailed study based on *A. lumbricoides* has yet not been undertaken, the detailed study of *A. lumbricoides* proteins may provide better vaccine candidates. The immune-informatics approach provides an opportunity for the evaluation of antigenic proteins as it involves bioinformatics, molecular biology, chemical engineering and mathematical modeling. It takes less time and cost and is more efficient than traditional methods.

Hence, in the current study, we aimed to construct a vaccine against *A. lumbricoides* using immune-informatics approach that can immunize human against ascariasis, thus giving a tool to health care workers to disrupt the life cycle of the parasite.

2. Materials and method

A sequential flow chart describing all procedures carried out for the construction of multi-epitope peptide vaccine for *A. lumbricoides* have been illustrated in (Figure 1).

2.1. Protein sequence retrieval and epitope prediction

Total proteome of *A. lumbricoides* was retrieved from the Wormbase parasite site version: WBPS15 (WS276) (https://parasite.wormbase.org/). This site comprises all the information related to the helminths till date [15]. Antigenic proteins that are recognized by B-cell receptor (BCR) and T-cell receptor (TCR) were identified by IEDB tools. The IEDB tool B-cell epitope prediction software BepiPred1.0 (http://tools.iedb.org/bcell/) works on the Hidden Markov Model (HMM) as well as Propensity scale method for B-cell epitope prediction [16]. T-cell epitopes were predicted by Net 2.3 and Net 4.0 servers provided by DTU bioinformatics (http://www.cbs.dtu.dk/services/). These servers work on Artificial Neural Networks (ANN) and sorts the predicted peptides based on the affinity to the MHCII-selected alleles. The DRB1*0701 and DRB1*1501alleles were reported to be expressed in case of ascariasis and same were used for this purpose [17,18].

2.2. *A. lumbricoides* membrane protein identification

The membrane proteins and excretory/secretory proteins are the first parasitic antigens that interact with the host immune system and induce an immunological response (activation/suppression). The signal peptides present on the N-terminal of proteins determine their fate to mark as secretory protein or to be located on the cell membrane. The Signal P 5.0 server (http://www.cbs.dtu.dk/services/SignalP/index.php) was used for this purpose [19]. The signal peptide prediction alone does not define the exact location of the proteins, so other servers were also used to find the membrane proteins, Deeploc server (http://www.cbs.dtu.dk/services/DeepLoc-1.0/index.php) and WolfPsort (https://wolfpsort.hgc.jp/), it predicted the subcellular localizations of proteins in the cell and the common membrane proteins thus identified were used for further analysis [20,21].

2.3. Elimination of conserve proteins within the Ascaris genus and human homologous proteins

To eliminate the conserved proteins present on related species or host homologs from above identified membrane antigenic proteins, the online available BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. The proteins which showed 50% similarity to human or *A. suum* proteins were filtered out and the proteins which were specific for *A. lumbricoides* only were selected for further studies[22–24].

2.4. Sorting of suitable vaccine candidates

To identify the most suitable and protective vaccine candidates, VaxiJen *v*2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was used with predefined threshold of 0.7, it works on alignment-independent mode [25]. To identify the non-allergic proteins, they were filtered using AlgPred (http://crdd.osdd.net/raghava/algpred/) and AllerTOP v. 2.0 (https://www.ddg-pharmfac.net/AllerTOP/) and non-allergic proteins were obtained. It works solely on auto cross covariance (ACC) transformation of protein sequences into uniform equal-length vectors of principal amino acids [26,27].

2.5. Identification of IFN-γ inducers and functional annotation of peptides

The predicated peptides were tested for IFN-γ stimulating ability using http://crdd.osdd.net/raghava/ifnepitope/predict.php server. This server is based on either IFN-g inducing and IFN-γ non-inducing datasets and generates all possible overlapping peptides from antigen. The IFN-γ positive epitopes were further evaluated for their toxicity by ToxinPred online server (https://webs.iiitd.edu.in/raghava/toxinpred/multi_submit.php). Both the servers are SVM and Motif-based [28] and the finally identified proteins were functionally annotated using BLAST server.

2.6. Constructing multi epitope vaccine

The T-cell peptide epitopes having IFN-gamma stimulating property along with B-cell epitopes thus identified based on allergenicity and antigenicity were joined using previously reported AAY and GPGPG linkers to create a fusion peptide [26]. To increase the immunogenic potential of the designed vaccine, a 50S ribosomal protein L7/L12 (UniProt accession no. P9WHE3) with EAAAK linker segment as an adjuvant was also added to N-terminal of peptide. The adjuvant sequence was obtained from UniPort, and it

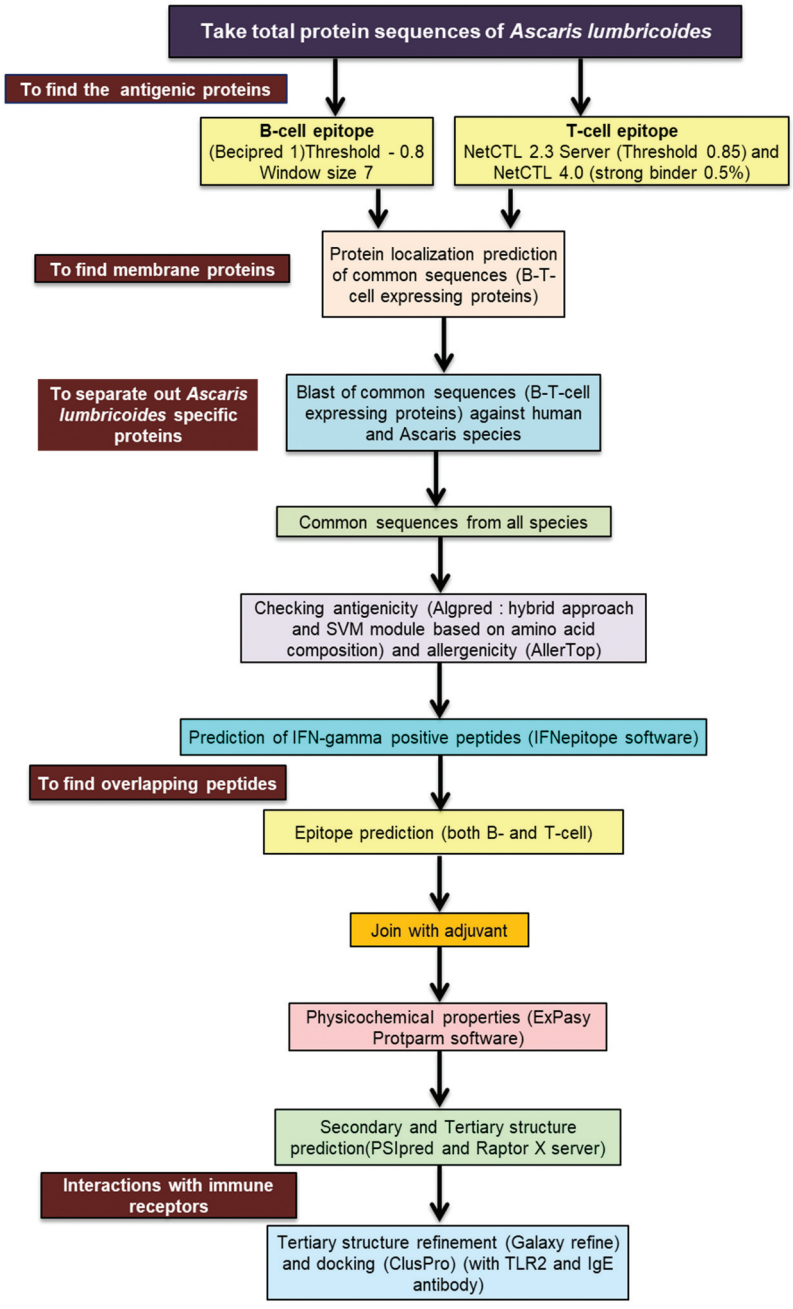

Figure 1. A detailed pipeline used to construct a multi-epitope peptide vaccine for *A. lumbricoides*.

comprised of 130 amino acids. It is a TLR4 agonist and facilitates the innate immune system activation by a vaccine.

2.7. Measuring constructed vaccine features

2.7.1. Physicochemical properties of the generated multi epitope vaccine

The physical and chemical properties of any protein is dependent on their amino acid sequence. ExPASY linked ProtParam server (https://web.expasy.org/protparam/) was used to predict these properties of the designed vaccine. It provides the mirror image of in-vivo characteristics of the designed vaccine. The ProtParam server computes several parameters such as theoretical pI, possible molecular weight, instability of the vaccine, possible half-life, aliphatic index and grand average of hydropathicity (GRAVY) [29].

2.7.2. Secondary structure prediction

The secondary structures of proteins are locally folded structures formed within the polypeptide, that include α-helix, β-sheets, and coils. These structures are stabilized by various chemical interactions such as hydrogen bonding, ionic bonding, and hydrophobic interactions. The secondary structure of the generated vaccine

was predicted by the PSIPRED tool (http://bioinf.cs.ucl.ac.uk/psipred/) which works on an artificial neural network [30,31].

2.7.3. Tertiary structure prediction and refinement

The 3-D structure of the peptide vaccine was predicted by Raptor X server, which is based on Deep learning (http://raptorx.uchicago.edu/) [32]. The tertiary structures should have the lowest energy level with proper bends and twists. The finest model generated by Raptor X was further refined by Galaxy Refine server, it properly rearranged the amino acids in the tertiary structure and stabilized the final structure [33]. The model generated by Galaxy refine server with lowest energy was again validated by Ramachandran plot using structure assessment service provided by Swiss Model (https://swissmodel.expasy.org/assess), it gave the Ramachandran plot of the entire structure [34]. To check the overall and local quality of the generated multi epitope vaccine ProSA web server was used (https://prosa.services.came.sbg.ac.at/prosa.php). The contact properties of the constructed vaccine were predicted by using Raptor X property prediction where solvent accessibility of the constituted amino acids was predicted [35].

2.8. Discontinuous epitope prediction

B-cell epitopes have been classified into two types, linear and discontinuous. Linear epitopes in the tertiary structure of protein attain exposed conformation to interact with B-cell receptors or immunoglobulins. To confirm this, discontinuous epitopes were predicted in the constructed multi epitope vaccine through Ellipro server (http://tools.iedb.org/ellipro). This server calculates ellipsoid score of individual residues. The residues with higher scores were correlated with better solvent approachability and these proteins were clustered based on the R distance [36].

2.9. Proteasomal cleavage prediction

Proteasomal cleavage peptides were predicted by using two software's 1. Proteasomal cleavage prediction server of Immuno-medicine Group and 2. Proteasomal Cleavage Prediction of IEDB, these are based on a neural network for the prediction of T-cell epitopes of the protein [37,38].

2.10. Docking of designed vaccine to immune receptors

TLR2 is reported to be highly expressed in human ascariasis infection [39–41], so constructed multi-epitope vaccine was docked with TLR2 on ClusPro 2.0 server (https://cluspro.bu.edu/). It gives top 10 docked models with different energy levels and the lowest energy docked complex have highest stability. High IgE antibodies are reported in the sera of patients, suggesting their main role against this parasite. Hence, docking study with IgE was also performed on the same server ClusPro 2.0 [42].

2.11. Validation of docking results by molecular dynamics

The stability of the docked complexes was validated by molecular dynamic (MD) simulation study as previously described [43–45]. In brief "by using protein-prep wizard the receptor-vaccine docked complexes were prepared, default parameters were used to fill the missing side chains, assign disulfide bonds, optimize hydrogen bond network, add missing hydrogen, and energy minimization by keeping the heavy atoms restrained at 0.30 Å. The MD simulation using the system builder of the Desmond Module 2.2 (www.schrodinger.com/desmond) was used with the prepared protein-vaccine complexes. The TIP4P water model and the salt concentration at 0.05 M was used to hydrate the model. The molecular dynamics simulation for 100ns was carried out using OPLS3e force field, NPT ensemble at 1.01 bar pressure and 300 K temperature with periodic boundary conditions. The Nose-Hoover chain thermostat method was used to carry out the experiments by keeping the relaxation time of 1ps, and Martyna-Tobian-Klienbarostat method by keeping the relaxation time of 2 ps. In MD simulations, standard deviation and fluctuation of the docked complex were demarcated in the form of Root mean square deviation (RMSD) and Root mean square fluctuation (RMSF).

2.12. In-silico cloning of the designed multiepitope vaccine

To make the designed multiepitope vaccine construct ready for the future large-scale production, in-silico cloning was performed. The amino acid sequence of the chimeric vaccine was back translated into nucleotide sequence by reverse translate tool of Sequence Manipulation Suite server (https://www.bioinformatics.org/sms2/rev_trans.html). Then codon optimization was done using Java Codon Adaption Tool (JCAT). The JCAT gives two outputs, Codon Adaption Index (CAI) with values between 0.2 and 1.0 and percentage GC content where GC content should be between 30% to 70%, respectively. After this it was in-silico cloned between *Hind*III and *EcoR*I site in pET28b vector to be ready for expression in bacterial system.

3. Results

3.1. Predicted epitopes of antigenic proteins

A total of 23,604 protein sequences of Ascaris were retrieved from WormBase ParaSite and 8436 and 16,612 proteins were identified to be having T-cell and B-cell epitopes, respectively. The predicted B-cell epitopes had threshold above 0.90 with 0.91% specificity, and only strong T-cell binders were chosen for DRB1*0701 and DRB1*1501 MHC II alleles interaction study.

3.2. *A. lumbricoides* membrane protein identification

Membrane proteins were identified by detecting the presence of signal peptide at N-terminal and a total of 383 epitope

expressing proteins were selected on this criterion. Out of these proteins, 175 membrane proteins were identified by two servers DeepLoc and WolfPsort and the proteins identified by both the servers were shortlisted for further studies. We could find specific function associated with 105 proteins, detailed list of these sorted proteins and their annotation is given in Supplement Table S1.

3.3. Identification of *A. lumbricoides* unique proteins

To minimize the after-effects and cross reactivity of the resultant vaccine, *A. lumbricoides* specific proteins were identified by doing BLAST against human and *A. suum* proteins. We found 143 and 199 proteins against human and *A. suum*, respectively. This also identified 127 unique membrane proteins, having B and T-cell epitopes specific to *A. lumbricoides* parasite and they were chosen for further analysis.

3.4. Identification of vaccine candidates

A vaccine should have high antigenicity and by setting 0.70 threshold value for Vaxijen, 10 highly antigenic proteins were sorted (Table 1). A suitable vaccine candidate should also be nontoxic, non-allergic and it should not cause any harmful effect to the host. Out of these 10 antigenic proteins, 8 were predicted to be non-allergic and nontoxic proteins by AlgPred and AllerTop v. 2.0 and used for further analysis (Table 1).

3.5. IFN-γ positive epitopes

The proinflammatory IFN-γ cytokine is released by T helper cells on their activation by MHC II binding peptides, it also induces B cells to generate antibodies and helps in parasite eradication [46,47]. So, the eight selected proteins were screened for their IFN-γ stimulating capability. We found seven of them (ALUE_0000893101, ALUE_0000996801, ALUE_0000995401, ALUE_0000852201, ALUE_0000898901, ALUE_0002090601 and ALUE_0001563101) having IFN-γ positive epitopes. The list of IFN-γ positive epitopes from these seven proteins is given in Table 2. All these peptides were again checked for their toxicity using ToxinPred server and were found to be nontoxic. These seven proteins were functionally annotated by using BLAST server to know their biological roles (Table 1) and majority of them were found to be integral membrane proteins and are associated with different types of membrane channels.

3.6. Assembling epitopes and construction of multi-epitope vaccine

Individual peptides have low immunogenicity as they are not easily recognized by the host immune system and are less stable because they can be easily degraded by host proteases, so the chosen peptides were linked with appropriate linkers to enhance their stability and immunogenicity. An adjuvant is also allied to further increase the immune-simulating property of the resultant vaccine.

For the building of multi-epitope vaccine candidate 13 T-cell, 11 B-cell epitopes and *M. tuberculosis* origin 50S ribosomal proteins as an adjuvant at N-terminal were assembled with AAY, GPGPG and EAAAK linkers, respectively. The list of epitopes identified and used for this purpose were given in Table 3. This 50s ribosomal protein is an agonist for TLR4 and stimulates the immune response of host and has been widely used as adjuvant [24–26]. The resultant vaccine contained 534 amino acids whose sequence is given below, and this sequence was used for further analysis.

Table 2. IFN-gamma positive epitope peptides.

MHC allele	Predicted peptide	Accession number
DRB1_0701	VQAWLGRTQSAAVKG	ALUE_0002090601
DRB1_1501	LLVVLPFVNLSSALI	ALUE_0002090601
DRB1_1501	AIVLIIASSYIAPSS	ALUE_0001563101
DRB1_1501	IAIVLIIASSYIAPS	ALUE_0001563101
DRB1_1501	LIAIVLIIASSYIAP	ALUE_0001563101
DRB1_1501	IVLIIASSYIAPSSC	ALUE_0001563101
DRB1_1501	SLIAIVLIIASSYIA	ALUE_0001563101
DRB1_1501	EGIFAMRRYTRARLR	ALUE_0000893101
DRB1_1501	IFAMRRYTRARLRKA	ALUE_0000893101
DRB1_1501	FAMRRYTRARLRKAT	ALUE_0000893101
DRB1_1501	RDLLANYSRLVRPVR	ALUE_0000893101
DRB1_1501	MVLVYHSDAHLLTPA	ALUE_0000852201
DRB1_1501	CFLMVLVYHSDAHLL	ALUE_0000852201
DRB1_1501	VLVYHSDAHLLTPAI	ALUE_0000852201
DRB1_0701	LVSCLKRTMSRVLII	ALUE_0000852201
DRB1_0701	VSCLKRTMSRVLIII	ALUE_0000852201
DRB1_0701	SCLKRTMSRVLIIIV	ALUE_0000852201
DRB1_0701	ELVSCLKRTMSRVLI	ALUE_0000852201
DRB1_0701	CLKRTMSRVLIIIVS	ALUE_0000852201
DRB1_0701	LKRTMSRVLIIIVSV	ALUE_0000852201
DRB1_0701	DGLYHGSSTSSLPSS	ALUE_0000898901
DRB1_0701	NMTFWSVRAVPDLVK	ALUE_0000996801
DRB1_0701	TAQATLLRVRTSIVL	ALUE_0000996801
DRB1_0701	IFLSRFLSTAQATLL	ALUE_0000996801
DRB1_0701	AQATLLRVRTSIVLN	ALUE_0000996801
DRB1_0701	RGDYAQALGNILQQT	ALUE_0000995401
DRB1_1501	ENPGGLYPNVPVADE	ALUE_0000995401

Table 1. Predication of antigenic, non- allergic and nontoxic protein.

Accession number	Score	Antigenic	Allergic	Toxic	Functions
ALUE_0002090601	0.8985	Probable ANTIGEN	Non-allergen	Nontoxic	Transthyretin-like family
ALUE_0000995401	0.7983	Probable ANTIGEN	Non-allergen	Nontoxic	TPM domain
ALUE_0001563101	0.7461	Probable ANTIGEN	Non-allergen	Nontoxic	Protein of unknown function, DUF255
ALUE_0001591601	0.7431	Probable ANTIGEN	Allergen	Nontoxic	
ALUE_0000893101	0.7163	Probable ANTIGEN	Non-allergen	Nontoxic	Neurotransmitter-gated ion-channel ligand binding domain
ALUE_0001551001	0.7156	Probable ANTIGEN	Non-allergen	Nontoxic	
ALUE_0000852201	0.7134	Probable ANTIGEN	Non-allergen	Nontoxic	Lung seven transmembrane receptor
ALUE_0000898901	0.7028	Probable ANTIGEN	Non-allergen	Nontoxic	Integral component of membrane
ALUE_0000996801	0.7021	Probable ANTIGEN	Non-allergen	Nontoxic	Integral component of membrane

Table 3. In seven highly antigenic protein, B and T-cell epitopes were predicted. Red, black and blue color showed the T-cell, B-cell and both epitopes, respectively.

S.no.	Accession no.	Epitopes (B and T-cell)
1.	ALUE_0000893101	RDLLANYSRLVRPVR MTPPTSE EGIFAMRRYTRARLRKA
2.	ALUE_0000996801	IFLSRFLSTAQATLLRVRTSIVLN NMTFWSVRAVPDLVK DEQGCESDAEDK ENEAPEEHD
3.	ALUE_0000995401	GNFPNPTTGD RGDYAQALGNILQQT ENPGGLYPNVPVADE
4.	ALUE_0000852201	CFLMVLVYHSDAHLLTPAI ELVSCLKRTMSRVLIIIVSV DDSEDENDEDE SAETRAESRRQKEAEQTDQK EEDKEQTE
5.	ALUE_0000898901	HPPQYRDDGLYHGSSTSSLPSS
6.	ALUE_0002090601	VQAWLGRTQSAAVKG LLVVLPFVNLSSALI
7.	ALUE_0001563101	SLIAIVLIIASSYIAPSSC EDDEEPEDERYSPDGE

MAKLSTDELLDAFKEMTLLELSDFVKKFEETFEVTAAAPVAVAAA-GAAPAGAAVEAAEEQSEFDVILEAAGDKKIGVIKVVREIVSGLGLKEA-KDLVDGAPKPLLEKVAKEAADEAKAKLEAAGATVTVKEAAAKRDLL-ANYSRLVRPVRAAYEGIFAMRRYTRARLRKAAAYIFLSRFLSTAQATL-LRVRTSIVLNAAYNMTFWSVRAVPDLVKAAYRGDYAQALGNILQQT-AAYENPGGLYPNVPVADEAAYCFLMVLVYHSDAHLLTPAIAAYELV-SCLKRTMSRVLIIIVSVAAYHPPQYRDDGLYHGSSTSSLPSSAAYVQA-WLGRTQSAAVKGAAYLLVVLPFVNLSSALIAAYSLIAIVLIIASSYIAPS-SCGPGPGMTPPTSEGPGPGDEQGCESDAEDKGPGPGENEAPEEHD-GPGPGGNFPNPTTGDGPGPGDDSEDENDEDEGPGPGSAETRAESR-RQKEAEQTDQKGPGPGEEDKEQTEGPGPGHTDETTPIDGPGPGDE-GRSDADEDDEEPEDERYSPDGE

3.7. Measuring constructed vaccine features

3.7.1. Calculating physicochemical properties of the vaccine

The physical and chemical properties of this vaccine was calculated by using the Protparam server, which included molecular weight (56,314.70 Dalton) and theoretical pI (4.52) of the assembled candidate vaccine. The estimated half-life of the vaccine 'in-vitro' in mammalian reticulocytes was calculated to be 30 h and classified as stable protein with instability index (II) of 37.41, low II means more stable protein (less than 40 is the criteria). The calculated aliphatic index defined the thermal stability of the vaccine which was 78.90 and the negative value of Grand average of hydropathicity (GRAVY) stated the hydrophilicity of the constructed vaccine was of −0.320. The detailed physio-chemical properties are given in supplementary table S2.

3.7.2. Prediction of secondary and tertiary structures

For the prediction of secondary structures in the constructed vaccine, PSIPRED server was used. It predicted that the multi-epitope vaccine has helix region mostly (50%), and remaining parts have β-sheet (10%) and coils (40%) as shown in Figure 2. The tertiary structure was predicted by using Raptor X server, it gave the top five models, and the first model has the highest estimated RMSD of 16.896 and was used for refinement.

3.7.3. Tertiary structure refinement and validation

The best model generated by Raptor X was refined by Galaxy Refine server, it gave top five models, and the first best model was chosen for refinement. Galaxy refinement analysis decreased the clash score from 306.4 to 20.5, GDT-HA from 1 to 0.9448, MolProbity from 4.867 to 2.144 and increased Rama favored amino acids from 92.7 to 96.1 of the initial model (Table 4) (Figure 3). The refined model was further validated by calculating overall and local quality score by ProSA web server, the Z-score of the refined model improved from −5.53 to 6.25 (Figure 4(A-B)). Ramachandran plot of any structural protein tells us about the torsional angles-phi and psi of the

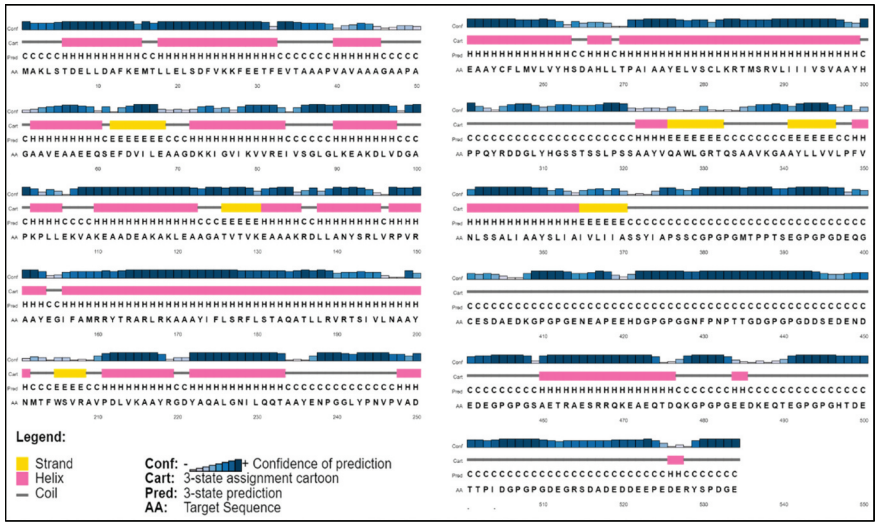

Figure 2. Secondary structure prediction of constructed vaccine using PSIPRED server. The graphical representation of purple and yellow color rectangles signifies the helix and beta-strands secondary structures whereas coils are represented as gray color line.

Table 4. Comparison of various parameters between initial designed vaccine model and final refined model.

Model	GDT-HA	RMSD	MolProbity	Clash score	Poor rotamers	Rama favored
Initial	1	0	4.867	306.4	85.3	92.7
Refined	0.9448	0.441	2.144	20.5	1.2	96.1

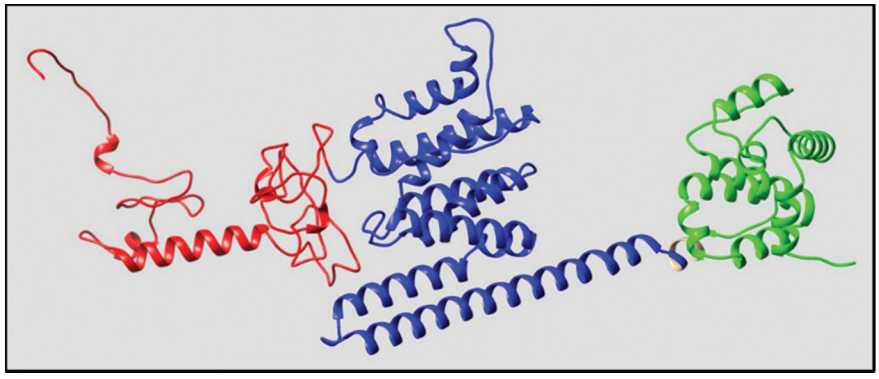

Figure 3. The final refined and validated tertiary structure of multiepitope vaccine. The different colors show different parts of the vaccine such as blue-T-cell epitopes, red-B-cell epitopes and green-Adjuvant.

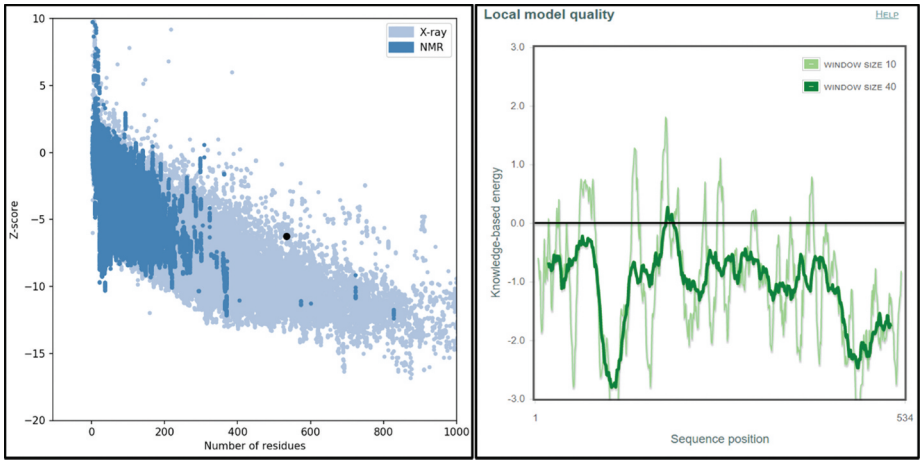

Figure 4. Overall and local quality of the final model was calculated using PROSA web server. 4A. The overall quality of the model is described in Z-score value and final validated modeled vaccine was having Z score of −6.25. 4B. Local quality tells about the energy level (entropy) of the protein structure and final modeled vaccine had less energy.

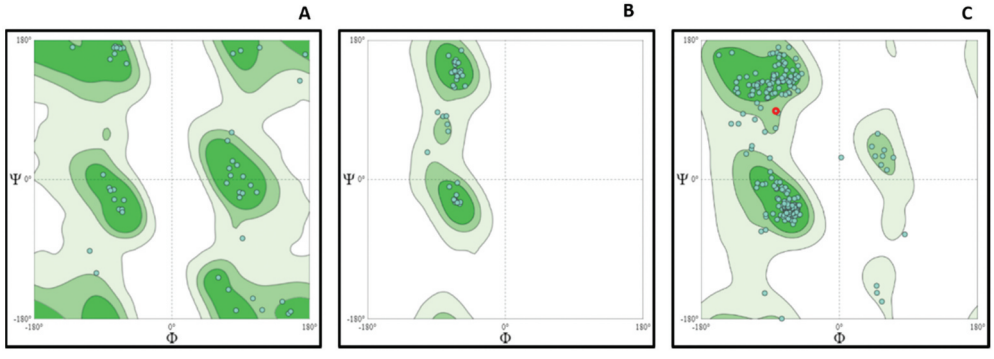

Figure 5. 5A. Ramachandran plot of constructed multiepitope vaccine described 96.05% amino acids lie in favored region, it showed the constructed multi-epitope vaccine had less steric hindrance. 5B. and 5 C. Separate Ramachandran plot of Glycine and Proline, respectively.

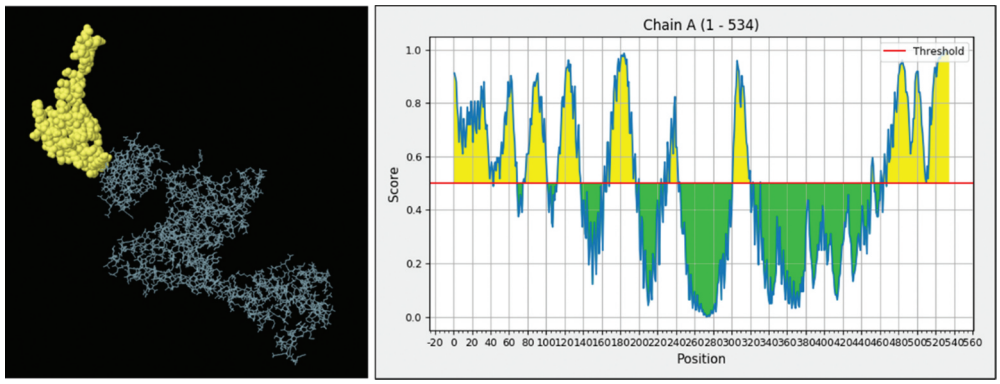

Figure 6. Prediction of B-cell binding epitopes of the constructed vaccine by discontinuous epitope predicting tool ElliPro by IEDB, this confirmed B-cell epitopes in the multi-epitope vaccine retain their potential, 6A. Yellow highlighted region showed B-cell epitopes, 6B. Individual potential of B-cell epitopes in the vaccine, above 0.5 threshold value.

residues and their distribution in a protein structure. It serves as an important indicator of three-dimensional structure quality and access to the stability of the proteins. Structure assessment service provided by Swiss Model calculated that 96.05% amino acids were present in the favored region (Figure 5(A-C)).

3.8. Structural B-cell epitope prediction

Pathogenic structural proteins directly interact with host immunoglobulins by binding on their paratopes, so the refined and validated vaccine model was checked for presence of discontinuous epitopes. Total 68 residues were found in the protein that were having more than 0.7 score (Figure 6(A)) and together these residues in 3-D protein structure acted as B-cell epitopes (Figure 6(B)).

3.9. Proteasomal cleavage prediction

The T-cells play a chief role in the activation of the host immune system, the antigen presenting cells present MHC I class bound peptides to cytotoxic T-cells and activate them. Proteasome is a protease complex that cleaves the ubiquitin tagged proteins in the cytosol, it has two subunits, a catalytic core particle (20S proteasome) and one or two terminal 19S regulatory particles. The proteasomal generated peptides have MHC I binding affinity and activate CD8 T cells. Using the IEDB Netchop server, we found 152 amino acids residues that can act as cleavage sites for proteasome in the validated model (Figure 7), whereas proteasome cleavage prediction server predicted 72 MHC I binding peptides. This validated that the constructed multi epitope vaccine could activate both, T helper as well as T cytotoxic cells.

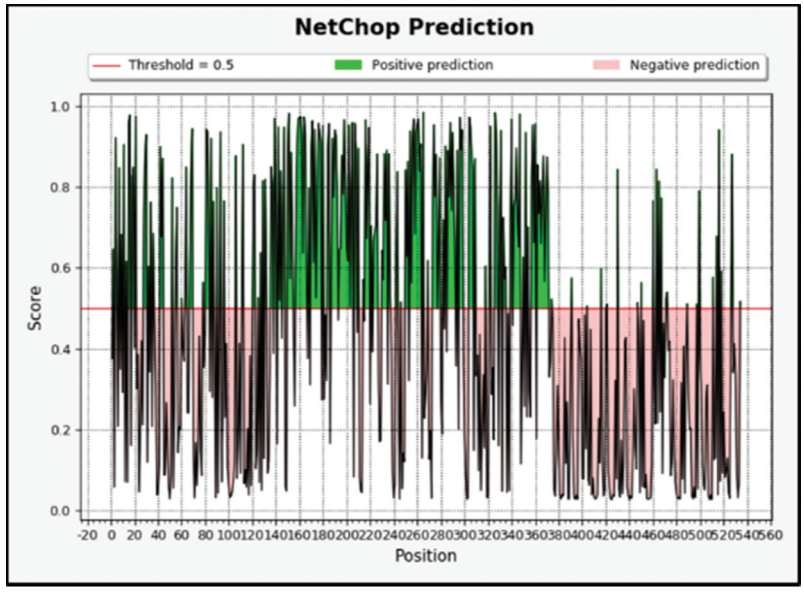

Figure 7. Prediction of positive proteasomal cleavage sites in the constructed vaccine using IEDB server. Green color bars represent positive sites for cleavage and 140 to 360 amino acid residues have high score than set threshold value.

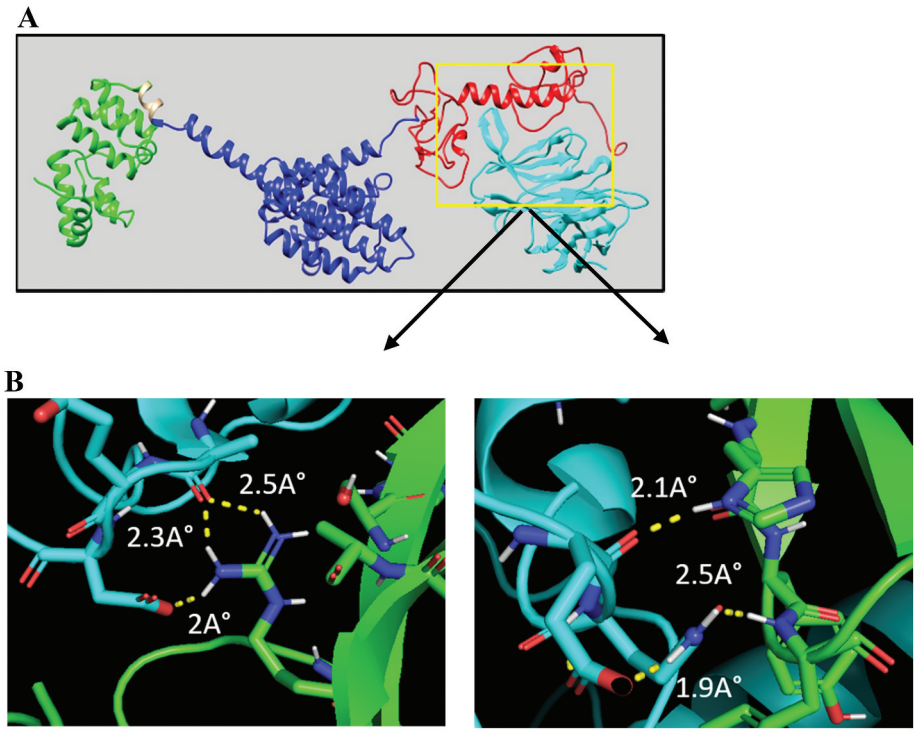

Figure 8. 8A. Constructed vaccine docked with Fab region of IgE antibody. 8B. Pymol software was used to visualize the interaction between docked complex and different length Hydrogen-bonds were visible in the docked complex. The length of H-bond was calculated and it vary from 1.8A° to 3A°.

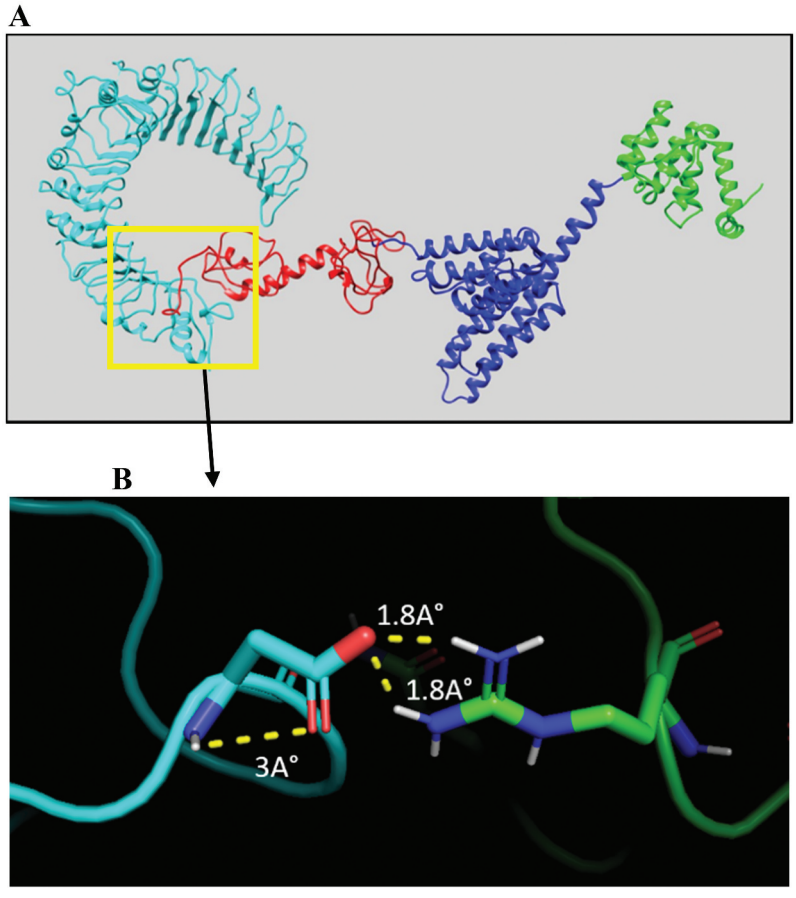

Figure 9. Constructed vaccine docked with Toll like receptor 2, an innate immune receptor.

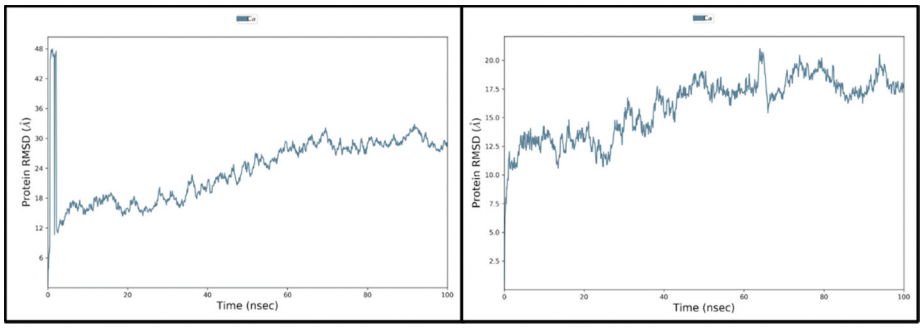

Figure 10. Validation of docking results by simulation and found interaction between constructed vaccine and 9A. IgE and 9B. TLR2 were stable over 100nsec. The average root mean square distance (RMSD) values of docked complex over 100ns showed little deviation in IgE docked vaccine complex, later on, it became stable and around 22A°. In TLR2 docked vaccine complex the average RMSD value was calculated around 15A°.

3.10. Molecular docking and molecular dynamic simulation

Innate immune cells are first to encounter pathogens and activate adaptive branch of the immune system. The constructed vaccines should form stable complex with different innate immune receptors called Pattern Recognition Receptors (PRR) such as Toll Like Receptors (TLRs) to activate the immune system. The TLRs recognize the pathogen-associated molecular patterns (PAMP) and activate immune system of host [39,40]. Apart from TLRs, Immunoglobulins (Ig) play an important role in eradication of any infection and are produced by activated B-lymphocytes [41]. TLR2 receptor and the Fab region of Immunoglobulin E was docked with validated model of the vaccine using ClusPro server [40,48,49]. This gave top 10 most stable interactive complexes with the first model having lowest binding energy (−610 kcal/mol and −453.6kcal/ml, respectively) and most stable interactions (Figures 8(A-B) and 9). The docking results were further validated by MD simulation studies, the complex (TLR2-designed vaccine) was stable for 100 nanoseconds at 37°C which confirmed their stability (Figure 10(A-B)). The radius of gyration of vaccine construct and complexes (IgE+vaccine and TLR2+ vaccine) was calculated. It varies from 32A° to 47A° in vaccine construct and it was observed that around 40nsec there was slight fluctuations, after that it was stable a at 100nsec. In complexes, it showed some variations from 50A° to 75A° in TLR2+ vaccine and 35A° to 62A° in IgE+vaccine (Supplementary Figure S1). Hydrogen bonds are prime interactions that provide stability to the construct; on an average 14 hydrogen bonds were calculated and it varied from 9 to 22 hydrogen bonds (Supplementary Figure S2).

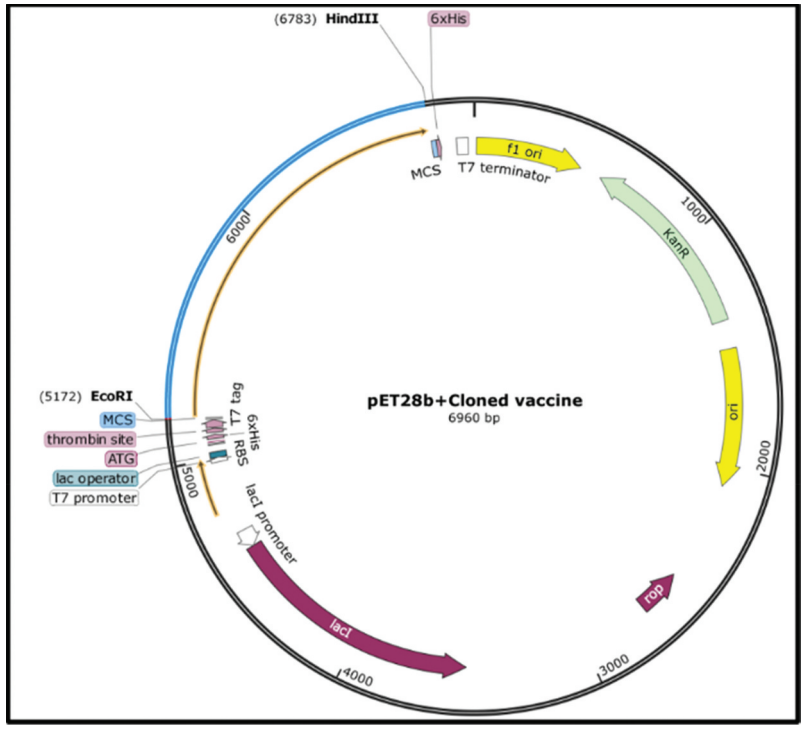

Figure 11. In silico cloning of multiepitope vaccine DNA optimized and cloned in pET28b vector. The vaccine was cloned in pET28b vector between EcoRI and HindIII restriction sites (shown in blue color).

3.11. In-silico cloning

Protein sequence of the designed multi-epitope vaccine was reverse transcribed into DNA and optimized for the K12 (*Escherichia coli*) strain. It improved the CAI-value of initial sequence from 0.577 to 1.0 and GC% content from 62.421 to 54.494%. The optimized DNA had 1691 nucleotides and was cloned between *Hind*III and *Eco*RI site in pET28b vector (Figure 11).

4. Discussion

Globally, the disease burden caused by *A. lumbricoides* is much higher than any other STH and there is no reliable vaccine available against this parasite. In the current study we used in-silico approache to design a multi-epitope vaccine candidate against *A. lumbricoides*. Not many efforts were made to control the spread of parasite due to lack of resources, awareness and its NTD tag. For better prophylactic measures developing better drugs and vaccines should be a priority for any disease, but it remains slow-paced for ascariasis owing to its NTD status. The immunoinformatics method is becoming quite popular and has been used to design candidate vaccine for various pathogens of viral, bacterial, fungal, and parasitic (intra/extra cellular) origin [44,50,51]. The renewed interest of scientists across globe in worms for their strong immuno-modulating properties and their potential in defining the outcome of other dreaded disease is also facilitating better understanding of helminths proteome [52].

Here for the first time, we have designed a multi-epitope vaccine against *A. lumbricoides* from the whole proteome database. From the proteomic database of Ascaris containing 23,604 proteins, 384 proteins were identified based on (i) high antigenicity with non-allergic properties (ii) their membrane localization and, (iii) ability to mount immune response (having B cells or T cells epitopes). Out of these 383 proteins further selection was done based on their membrane localization, uniqueness to species, antigenicity, toxicity and IFN inducing capability. That gave us finally seven peptides and they were assembled to make a chimeric multiepitope vaccine stitched together with linkers and adjuvant. Use of membrane proteins to develop multiepitope vaccine is well documented and recently same approach has been utilized by Sanches RCO et al (2021) to develop vaccine candidate for Schistosoma [53]. In our earlier study too, we used the same strategy to come with a candidate vaccine for another helminth *Taenia solium* [43]. An ideal multi-epitope vaccine should contain both B- and T-cell epitopes from pathogen for its higher efficacy and should be linked with suitable adjuvant at N-terminal to increase the immunogenic potential of the vaccine. We followed the same strategy to increase the ability of the designed vaccine. From these unique proteins we identified 13 T-cell and 11 B-cell epitopes (Table 4), these small epitopes individually cannot mount a strong immune response due to their low molecular weight hence they were connected with linkers AAY, GPGPG and EAAAK. These linkers also help to enhance the stability of the constructed vaccine. Further to enhance the immunogenicity of this multiepitope vaccine we added *M. tuberculosis* origin 50S ribosomal proteins as an adjuvant at N-terminal. The same adjuvant has been used by other groups too for its ability to activate immune system as it directly activates TLR4 signaling [24,26]. For the secondary and tertiary structure prediction of any protein, different online tools are available, which majorly work on homology modeling and for our study we had used highly referred software's such as PSIPRED and RaptorX for secondary and tertiary structure prediction, respectively. Predicted structure of the designed vaccine was further refined using 3-D refinement and Galaxy refine tools; this contributes to the structure by filling gaps and provides more stability to protein. Once the proteins are inside the antigen presenting cells (APC), they are subjected to proteolytic attack, cleaved, and then presented on APCs along with MHC molecules. So, we did proteasomal cleavage prediction of the construct and found 72 MHC I binding peptides along with 152 probable cleavage sites. This confirmed that after processing of this chimeric multiepitope vaccine protein inside the cell, the epitopes needed for T cell activation will be generated. This also validated the linkers and their position in the vaccine construct (Figure 6).

Innate immune response has a major role in the elimination of any infection and TLRs are primary innate receptors that interact with parasitic proteins, to evaluate the immunogenicity of multi-epitope vaccine, it was docked with the human TLR2, which had been reported to be over expressed in patients during Ascaris infections [41,54]. To analyze the efficacy of designed vaccine, molecular docking was performed with immune receptors TLR2 and IgE, it was done using ClusPro (Figures 7(A-B) and 8). Molecular dynamic simulation studies were carried out to further confirm the interactions between designed vaccine and immune system receptors. The Desmond Module over 100ns was used to determine stability of the final complex and it was found to be stable over 100ns time thus confirming its robustness for further use (Figure 9(A-B)). This result gave strength to our study as to qualify for being an effective vaccine, the vaccine candidate should be able to bind with PRRs to stimulate downstream signaling in immune cells. The translation effectiveness of the final vaccine was determined by Java Codon adaptation tool.

The immunoinformatic approach of vaccine design addresses and meets the challenges and constraints of the traditional vaccine design in terms of cost, time and resource optimization and thus has gained significant attention from scientific community. It's the need of hour, specially for tropical neglected diseases which do not receive the due amount of attention in comparison to their enormous impact on population well-being. By using various existing bioinformatics tools (primarily free software), we designed a potential multi-epitope chimeric subunit vaccine, displayed its ability to stimulate and sustain strong immune responses involving both the arms of immunity. The designed vaccine construct was further evaluated for various parameters like physiochemical parameters, allergenicity, antigenicity and efficiency of forming stable complexes with immune receptors and it is found to meet criteria for all these parameters. We also did codon optimization to purify designed vaccine in bacterial system followed by in-silico cloning of the final cloned construct to make it ready for expression and purification.

5. Conclusions

Although the multiepitope vaccine we designed from proteome of Ascaris is all in-silico, but this study can provide an elementary ground to researchers to apply this approach to design a vaccine in a wet lab and take it further for in-vitro studies. Therefore, with this study we highlight the usefulness of immnuoinformatics approach in addressing requirements of an NTD in resource efficient manner and suggest its applicability in future vaccine designs. We put forward a potential multi-epitope vaccine candidate against ascariasis capable of eliciting a strong immunological memory response, ready for expression in prokaryotic system for subsequent in vitro and in vivo validation experiments.

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Declaration of interest

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Authors contributions

RK, NA, SKR, NS, SKS, PK & AKK performed experiments. RK, NA, SSR, AKK, AM, AP wrote manuscript. AM &AP critically evaluated the data and manuscript. AP conceived, designed and over all monitored the work.

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