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In Silico Design and Evaluation of a Novel Multi-Epitope Vaccine Against *Treponema denticola* (strain ATCC 35405): A Reverse Vaccinology Approach

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Abstract

Treponema denticola is a gram-negative, drug resistant bacterium found in primary dentition infections and around teeth, causing inflammation and tissue homeostasis linked to periodontal diseases including early-onset periodontitis, necrotizing ulcerative gingivitis, and acute pericoronitis. In previous research, potential drug targets against the pathogen had been identified and characterized. However, as of now, there are no vaccines specifically available against *T. denticola* for human use. Hence, this study aims to design an immunoinformatic vaccine using a reverse vaccinology approach against this deadly pathogen. Human (HLA) allele specific potential T-cell and B-cell epitopes were shortlisted through a stringent procedure from a total of two antigenic proteins of *T. denticola* (namely, Acetate kinase AckA and Chromosomal replication initiator protein DnaA). The multi-epitope vaccine (MEV) was constructed using linkers with adjuvant human beta-defensin-3. The vaccine construct was verified, based on its antigenicity and physicochemical properties, and showed strong binding with the human receptor (TLR4). Molecular dynamics simulation confirmed the stable nature of the docked complex. The results of *in silico* immune simulation revealed that constructed vaccine activated B and T lymphocytes which induced high levels of antibodies and cytokines. Furthermore, *in silico* cloning analysis ensured efficient expression of desired gene in the microbial system. Thus, the vaccine construct developed in this study is capable of eliciting both innate and adaptive immunity and has all the potential for the development of a next-generation vaccine which may in turn effectively combat the periodontal pathogen *T. denticola.* Nevertheless, further *in vitro* and animal studies are warranted to strengthen our findings for its utility as a probable preventive measure.

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Introduction

Treponema denticola is a gram-negative, obligate anaerobic, motile, and highly proteolytic spirochete bacterium with four periplasmic flagella, which facilitate its motility even in a viscous environment.[1] It is often seen in primary dentition infections and in pockets around teeth which is intimately linked to periodontal diseases such as early-onset periodontitis, necrotizing ulcerative gingivitis, and acute pericoronitis.[2] Periodontal diseases in the oral cavity are caused by the growth and buildup of bacteria resulting from inadequate oral hygiene.[3] Both basic studies and clinical data indicate that the abundance of *T. denticola*, along with other proteolytic gram-negative bacteria, in significant quantities inside periodontal pockets, likely has a substantial impact on the development of periodontal disease.[4] However, the precise impact of the pathogen in causing disease still requires more investigation and evidence.[5] Periodontal diseases affect about 5 to 20% of the worldwide population and are amongst the most common chronic infections.[6] Thus, to prevent periodontal diseases and control infections, the development of vaccines against *T. denticola* is one of the most important strategies. Till now, no vaccines are available against this periodontal pathogen.[7,8] While there are many approaches for vaccine development like killed vaccine, live-attenuated vaccine, DNA/RNA vaccines, etc., multi-epitope based or subunit vaccines have advantages over them. This is

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because an ideal multi-epitope vaccine (MEV) can enhance immune response and eventually scale down the risk of re-infection by magnifying the host immunogenicity.[9] Moreover, they are safer as they do not require an entire pathogen. Also, highly promiscuous epitopes can bind multiple alleles simultaneously which can ensure the desired immune response among a heterogeneous human population. As they contain multiple MHC restricted epitopes, they can be recognized by TCRs of various T cell subsets. Along with inducing strong cellular and humoral immune responses with the help of the combination of multiple kinds of epitopes, they also can enhance long-lasting immune responses with the help of their adjuvant.[10] Several studies have highlighted the use of reverse vaccinology approach for the construction of multi-epitope vaccines against various pathogens.[11-14] Our aim is to design a multi-epitope based vaccine by screening target proteins in *T. denticola* (strain ATCC 35405) so that the most immunogenic peptides can be used. In this study, the analysis was done using the identified and characterized essential non-homologous proteins of *T. denticola* obtained from previous research.[15] We strongly believe that our findings will provide enough information and better guidance for the vaccine construct and for further vaccine developments. Furthermore, our findings predict that the epitopes from the target proteins serve

the multi-epitope based vaccine and have the potential to elicit immune responses. Therefore, it may be used for the treatment of the periodontal infections caused by the pathogen *T. denticola*.

Methods

The vaccine candidate for *T. denticola* (strain ATCC 35405) was predicted using the reverse vaccinology approach. The methodology for developing a multi-epitope peptide based vaccine using the reverse vaccinology approach is presented in Figure 1.

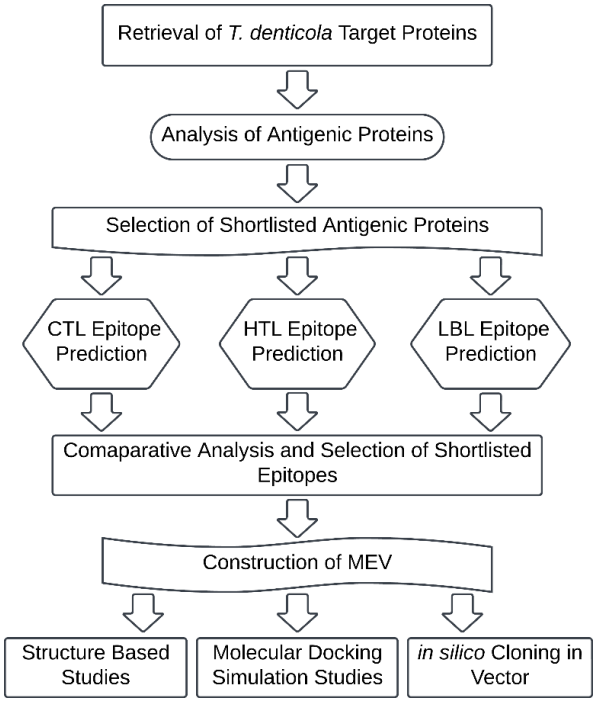

Fig. 1: Overall flowchart for the reverse vaccinology study

Retrieval of Protein Sequences: The target proteins of *T. denticola* selected for this study was obtained from previous research titled, "*In silico* Identification and Characterization of Novel Drug Targets in *Treponema denticola* (strain ATCC 35405 / DSM 14222 / CIP 103919 / JCM 8153 / KCTC 15104): A Subtractive Genomics Approach".[15] Then the selected proteins were retrieved from UniProt database[16–18] and downloaded in FASTA format for further analysis.

Identification of Most Potent Antigenic Proteins: To identify the most potent antigenic proteins out of the selected 11 essential non-homologous proteins of *T. denticola*, we utilized the online prediction server VaxiJen2.0[19] with a threshold of 0.4. The server employs a novel alignment-independent method, which is based on auto cross-covariance (ACC) transformation of protein sequences into uniform vectors of principal amino acid properties. Depending on the target organisms (bacterial, viral, and tumor protein datasets), the server accuracy varies from 70 to 89%.[19] The proteins having the highest antigenic value was considered as the most potent antigenic proteins and were selected for further analysis.

Prediction of CD8+ Cytotoxic T cell (CTL) epitopes: For the prediction of cytotoxic CD8+ T cell (CTL) epitopes, NetCTL 1.2 server[20] was used for the selected antigenic proteins of *T. denticola*. The candidate epitopes were predicted based on the MHC class I supertypes (A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, and B62) and peptide binding. The threshold value was set to 0.75 by which we could assess our findings more decisively to generate more epitopes. Afterwards, NetCTL1.2 predicted epitopes of the proteins were then subjected to IEDB MHCI Immunogenicity prediction tool[21], which determined the immunogenicity scores of each predicted epitope. The epitopes having positive scores were selected and the rest were discarded. The immunogenic epitopes were then subjected to the MHC Class I peptide binding tool of IEDB[22], using the NetMHCIpan 4.1EL as the selected method, for the identification of epitopes

which were bound to more than or equal to ≥4 MHC Class I alleles of humans, and the epitopes were selected based on the percentile rank which were below or equal to 1% to filter out the peptide-alleles with weak binding affinity. The selected CTL epitopes were checked for antigenicity by VaxiJen2.0[19], with the threshold value set at 0.4, allergenicity by AlgPred2[23], with the threshold value set at 0.3 and toxicity by ToxinPred2[24], with the threshold value set at 0.6. The epitopes having met the criteria were selected for the construction of the multi-epitope vaccine.

Prediction of CD4+ Helper T cell (HTL) epitopes: The peptide binding to MHC class II molecules tool of IEDB was used to predict T-helper cell (HTL) epitopes for the two antigenic proteins.[25,26] The IEDB recommended 2.22 method was used as the prediction method using specific MHC class II alleles.[27] The peptide length was selected as 15 mer and a percentile rank with a threshold of 25% was used for MHC class II binding epitopes to filter out peptide-allele with weak binding affinity. The IEDB 7-allele HLA reference set was used and peptides with the number of binding alleles ≥4 for MHC class II binding epitopes were selected for further analyses. The selected HTL epitopes were analyzed for their antigenicity, allergenicity, and toxicity using VaxiJen v2.0, AlgPred2, and ToxinPred2, respectively. MHC Class II epitopes that passed antigenicity, toxicity, and allergenicity tests were further analyzed for Interferon-γ production using IFNepitope, for IL-4 production using IL4pred, and for IL-10 production using IL-10Pred.[28-30]

Prediction of Linear B cell epitopes: The amino acid sequences of the two selected antigenic proteins were then analyzed for linear B cell (LBL) epitope prediction using BepiPred-2.0 of the IEDB server[31] with the threshold value of 0.5 and length greater than 8AA. Along with this, the predicted epitopes were further subjected for transmembrane topology prediction, utilizing the TMHMM v2.0 server.[32] In addition, all the selected epitopes were evaluated by IgPred server[33] for IgG, IgM, and IgA inducibility properties. Finally, all the predicted epitopes were analyzed for their antigenicity, allergenicity and toxicity using VaxiJen v2.0, AlgPred2, and ToxinPred2, respectively.

Vaccine Constructionn: The pre-selected B and T cell epitopes were used for the multi-epitope vaccine (MEV) construction.[13,34,35] Three linkers KK, GPGPG, and AAY were used to link the epitopes together. The GPGPG linker was used to connect the MHC Class II epitopes, KK linker was used to link the linear B cell epitopes, and the AAY linker was used to connect the MHC Class I epitopes. Moreover, at the N terminal, the Human Beta-defensin 3 (UniProt ID Q5U7J2_HUMAN) was linked using EAAAK linker as an adjuvant for boosting of the immunogenicity of the vaccine. Finally, to enable protein purification and identification, 6xHis tag was added at the C-terminal end.[36]

Physiochemical Property Analysis of Vaccine Construct: The vaccine that was finally developed was subjected to antigenicity, toxicity, and allergenicity prediction using VaxiJen v2.0, ToxinPred2, and AlgPred2, respectively.[19,23,24] Also, the construct sequence was submitted to Expasy ProtParam tool to calculate different parameters such as the number of amino acids, molecular weight, theoretical isoelectric point (pI), half-life, instability index, aliphatic index, GRAVY score, etc.[37] Based on the physicochemical properties of the construct, the quality of the

vaccine was assessed.

2D Structure Prediction: Protein secondary structure plays an important role in protein folding properties and its physicochemical characteristics.[38] Thus, for the prediction of the constructed MEV secondary structure, the PSIPRED server was used.

3D Structure Prediction, Refinement, and Validation: The three-dimensional structure of the constructed MEV was predicted using the I-Tasser tool.[39] Because of the lack of any suitable template for homology modelling, this ab-initio approach was used. Moreover, for the refinement of the predicted protein structure, the GalaxyWEB server was employed.[40] Following the refinement, the models were subjected to quality checking and assessment using SAVES v6.1, ERRAT, ProSAweb, and the Ramachandran plot analysis using SWISS-MODEL Structure Assessment platform.[41-44] Based on these results, the qualities of the refined models were validated and the most suitable model was selected for further steps.

Discontinuous B-cell epitopes of the constructed vaccine: To predict conformational B cell epitopes on the MEV construct, ElliPro[45] was used. The PDB structure of the constructed MEV was submitted to the server with a threshold value of 0.5. The residues predicted as conformational B cell epitope by the tool were selected and visualized using PyMOL v3.1.[46]

Molecular Docking Analysis and Binding Affinity Prediction of the Vaccine Construct: To assess the binding affinity of the MEV construct with TLR4 (PDB ID: 4G8A), molecular docking was performed using ClusPro2.0 protein-protein docking server.[47] Furthermore, the vaccine-receptor complex was subsequently analyzed for binding affinities utilizing the PRODIGY web server, which provided the ΔG values of the vaccine-TLR4 complex.

Molecular Dynamic Simulation of the Vaccine and TLR4 complex: The binding stability and flexibility of the vaccine and TLR4 complex was analyzed using iMODS server which performs Normal Mode Analysis (NMA) in internal (dihedral) coordinates using an elastic network model (ENM).[48] The server also provides B-factor and deformability plots, covariance map, mode variance plot, eigenvalues and elastic network.

In silico Immune Simulation: The C-ImmSimm server was used to perform an *in silico* immune simulation to estimate the real-life immunogenic and immune response of the designed peptide vaccine.[49] The server is an agent-based model that uses position-specific scoring matrices (PSSM) derived from machine learning techniques for predicting immune interactions. According to the most currently used vaccines, the minimum recommended interval between the first and second doses is 4 weeks.[50] Three doses of injection, each containing 1000 antigen proteins and no LPS, were administered at 1, 84, and 168 time-steps. The other parameters, including random seed, simulation volume, and simulation step were kept at 12345, 10 µl, and 1050, respectively.

Codon Optimization of the Vaccine and in silico Cloning: The Java Codon Adaptation Tool (JCat)[51] was used to get the optimized DNA sequence of the final vaccine construct along with the codon adaptation index (CAI) value and GC content. SnapGene software (version 8.0.1) was used to integrate the adapted DNA sequence into a suitable vector.

Results

In the current study, the reverse vaccinology approach was utilized, which resulted in the identification of potential vaccine candidates along with the vaccine construct against *T. denticola*.

Retrieval of Protein Sequences: The 11 target proteins of *T. denticola* selected for this study were obtained from previous research[15], and were retrieved from the UniProt database and then stored in FASTA format for further analysis.

Identification of Most Potent Antigenic Proteins: Out of the 11 selected target proteins of *T. denticola,* 8 proteins were predicted to be antigenic, which are listed along with their UniProt accension numbers, antigenic prediction results, and amino acid numbers in Table 1, along with the two most antigenic proteins selected for *in silico* vaccine development (Table-I).

Table-I: List of antigenic proteins of *T. denticola.*

Sl. No	*T. denticola* Proteins	UniProt ID	Antigenic Value	VaxiJen2.0 Result	Length AA
1	***Chromosomal replication initiator protein DnaA***	***O87546***	***0.5902***	***Antigenic***	***469***
2	***Acetate kinase***	***Q73P66***	***0.5745***	***Antigenic***	***397***
3	Trans-2-enoyl-CoA reductase	Q73Q47	0.5526	Antigenic	397
4	Protein RecA	P62221	0.5403	Antigenic	414
5	Ribonuclease Y	Q73R54	0.5075	Antigenic	509
6	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Q73Q03	0.4929	Antigenic	426
7	UvrABC system protein C	Q73JT1	0.4398	Antigenic	652
8	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	Q73Q55	0.4038	Antigenic	305
9	Glycogen synthase	Q73MC7	0.3847	Non-Antigenic	491
10	Fructose-bisphosphate aldolase class 1	Q73QV3	0.2809	Non-Antigenic	295
11	Pyridoxal 5'-phosphate synthase subunit PdxS	Q73QI7	0.1998	Non-Antigenic	282

Prediction of CD8+ Cytotoxic T cell (CTL) epitopes: A total of 272 CTL epitopes were predicted from NetCTL 1.2. Of them, 160 epitopes were predicted to be positive in terms of immunogenicity by the IEDB class I immunogenicity tool. Moreover, 68 epitopes out of the 160 epitopes, had a percentile rank of ≤1% and had bound to ≥4 MHC Class I alleles as predicted with MHC class I tool of IEDB. Out of 68 epitopes, 18 epitopes satisfied the antigenicity, allergenicity, and toxicity tests. Finally, 8 epitopes out of 18 antigenic epitopes were selected for vaccine construction (Table-II).

Prediction of CD4+ Helper T cell (HTL) epitopes: A total of 656 HTL epitopes were predicted by MHC class II tool of IEDB. Of them, 77 epitopes had bound with ≥4 MHC Class II alleles. Moreover, 30 out of 77 epitopes, passed

the antigenicity, allergenicity, and toxicity tests. After considering the IFN-γ, IL-4, and IL-10 inducibility utilizing IFNepitope, IL4pred, and IL10pred servers, respectively, 8 out of 30 epitopes were selected for vaccine construction (Table-II).

Prediction of Linear B cell epitopes: Bepipred2.0 Linear Epitope Prediction tool predicted 15 LBL epitopes of variable length ranging from 8 to 64 amino acids. Among them, 10 epitopes were predicted to be antigenic, non-toxic, and non-allergen by Vaxijen2.0, ToxinPred2, and AlgPred2 respectively. Following that, four epitopes belonged to the outside region according to the TMHMM2.0 output. Furthermore, two epitopes were recognized as IgG-epitopes by the IgPred server. After further assessment of surface accessibility, hydrophilicity, flexibility, antigenicity, containing a beta turn in the secondary structure, allergenicity, toxicity, transmembrane topology, and IgG inducibility, eight epitopes were selected as the potential linear B cell epitopes (Table-II).

Vaccine Construction: A total of 24 epitopes- 8 LBL, 8 MHC Class II, and 8 MHC Class I epitopes were used to design the MEV. The predicted epitope sequences were joined using GPGPG, AAY, and KK linkers. The adjuvant Human Beta-defensin 3 was attached to the N terminal with the help of EAAAK linkers. Also, to facilitate protein purification, a 6xHis tag was added to the C terminal end. Thus, the final construct generated is shown in Figure 2C (Bold letters indicate the linkers).

Physiochemical Property Analysis of Vaccine Construct: The results of antigenicity, allergenicity, toxicity, and physiochemical properties analyses determined the vaccine to be safe and suitable for the human usage. VaxiJen2.0 predicted the vaccine to be antigenic with a score of 1.0093, AlgPred2 projected the vaccine to be non-allergen, and ToxinPred2 evaluated the vaccine to be non-toxic. The physicochemical properties along with the antigenicity, allergenicity, toxicity assessment of the vaccine construct is listed in Table-III.

2D Structure Prediction: The prediction of the vaccine's secondary structure was carried out utilizing the PSIPRED server. The PSIPRED server provided the characteristics of the vaccine's secondary structure through three-state prediction, including coil, helix, and strands (Fig. 2B).

3D Structure Prediction, Refinement & Validation: The 3D model of the final vaccine was built by the I-Tasser web server; five models were generated. The best model was chosen based on the higher quality of the estimated TM-score. Following this, the refined 3D model was retrieved from the GalaxyWEB (Fig. 3A) and afterwards validated using SAVES v6.1, ERRAT, and ProSA-web servers, as shown in (Fig. 3B). The final selected 3D model had a ERRAT value of 72.5055 (Fig. 3C) and a Verify3D score of 85.36% (Fig. 3E). The Ramachandran plot analysis of the final model by SWISS-MODEL Structure Validation platform revealed that 86.16% of residues are located in the most favoured regions (Fig. 3B). Moreover, the Z-score calculated by the ProSA-web server was – 5.37 (Fig. 3D).

Discontinuous B-cell epitopes: Out of 485 total residues, ElliPro predicted 272 residues to possess discontinuous epitope qualities (Table-IV). The ElliPro score of these residues ranged between 0.601 and 0.871.

Table-II: The list of selected epitopes for vaccine construction

CTL Epitopes	Length	Antigenic Score	Immuno-genic Score	IFN-γ	IL-4	IL-10	Topo-logy	IgG	Toxicity	Allergen	Protein
MVAVFDTAF	9	0.4565	0.24474	N/A	N/A	N/A	N/A	N/A	Non-Toxic	Non-Allergen	AckA
DLDPAILPF	9	1.2872	0.14358	N/A	N/A	N/A	N/A	N/A	Non-Toxic	Non-Allergen	AckA
YIYALPYEY	9	1.3656	0.07231	N/A	N/A	N/A	N/A	N/A	Non-Toxic	Non-Allergen	AckA
AQLGGVDAL	9	0.7354	0.14394	N/A	N/A	N/A	N/A	N/A	Non-Toxic	Non-Allergen	AckA
SAYNPFLIY	9	0.9920	0.16009	N/A	N/A	N/A	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
EIRYAILLK	9	0.9278	0.16062	N/A	N/A	N/A	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
FVVGPNNNF	9	0.4213	0.02137	N/A	N/A	N/A	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
SEWDYKIFW	9	1.7274	0.06834	N/A	N/A	N/A	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
HTL Epitopes											
KYIASYAAQLGGVDA	15	0.6327	N/A	Non	Non	Non	N/A	N/A	Non-Toxic	Non-Allergen	AckA
PAILPFIMNKEKLSA	15	0.4111	N/A	Non	Yes	Yes	N/A	N/A	Non-Toxic	Non-Allergen	AckA
DLIQRTVADYFSISI	15	0.5067	N/A	Yes	Yes	Non	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
EWDYKIFWDEAVNQF	15	0.8330	N/A	Non	Yes	Non	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
IEKKLFELSGKKISI	15	0.7094	N/A	Non	Yes	Non	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
KSTENTVYLSVPSKF	15	0.5073	N/A	Non	Yes	Yes	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
NQFKEELAFSIFSMW	15	0.5974	N/A	Non	Yes	Non	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
VADYFSISISDIKSK	15	1.1117	N/A	Non	Yes	Yes	N/A	N/A	Non-Toxic	Non-Allergen	DnaA
LBL Epitopes											
IGIEGSRIKHEKKGM DAVLIEEKMNDHKR	29	0.9998	N/A	N/A	N/A	N/A	Inside	Yes	Non-Toxic	Non-Allergen	AckA
EKNKVRGKLTDAS	13	0.9822	N/A	N/A	N/A	N/A	Inside	Non	Non-Toxic	Non-Allergen	AckA
NHGVVKSM	8	0.7127	N/A	N/A	N/A	N/A	Inside	Non	Non-Toxic	Non-Allergen	AckA
GGEEFNKS	8	0.9781	N/A	N/A	N/A	N/A	Outside	Non	Non-Toxic	Non-Allergen	AckA
KPNTSEDLSKAENE GGNDKKEDAAKPSS AESKKKSVKTEGGR GQHPDLRPEYNFED FVVGPNNN	64	1.5665	N/A	N/A	N/A	N/A	Outside	Yes	Non-Toxic	Non-Allergen	DnaA
EEQIAADPSLEK	12	0.7557	N/A	N/A	N/A	N/A	Outside	Non	Non-Toxic	Non-Allergen	DnaA
GNDFGGRD	8	2.4867	N/A	N/A	N/A	N/A	Outside	Non	Non-Toxic	Non-Allergen	DnaA
ISISDIKSKKRT	8	1.1094	N/A	N/A	N/A	N/A	Inside	Non	Non-Toxic	Non-Allergen	DnaA

Table-III: Physiochemical properties of the vaccine construct

Property	**Value**
Number of amino acids	485
Molecular weight	53797.19
Theoretical pI	9.26
Chemical Formula	$C_{2438}H_{3744}N_{648}O_{705}S_{12}$
Instability index	31.40
Aliphatic Index	64.85
Grand average of hydropathicity (GRAVY)	-0.653
Total number of negatively charged residues (Asp + Glu)	57
Total number of positively charged residues (Arg + Lys)	76
Total number of atoms	7547
Antigenicity	1.0093
Allergenicity	Non-allergen
Toxicity	Non-toxic
Estimated half-life in mammalian reticulocytes, in vitro	1 hour
Estimated half-life in yeast, in vivo	30 mins
Estimated half-life in Escherichia coli, in vivo	>10 hours

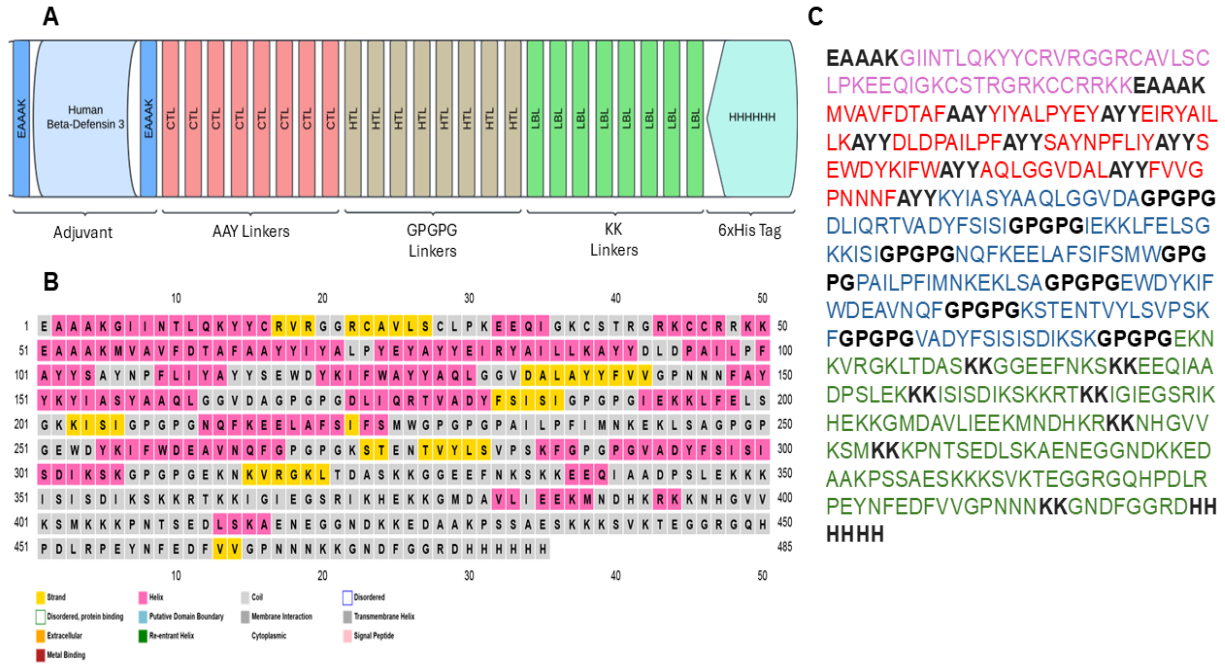

Fig. 2: Linear representation of the vaccine construct: (A) Graphical representation (B) The predicted 2D structure (C) The amino acid sequence along with the linkers and 6xHisTag.

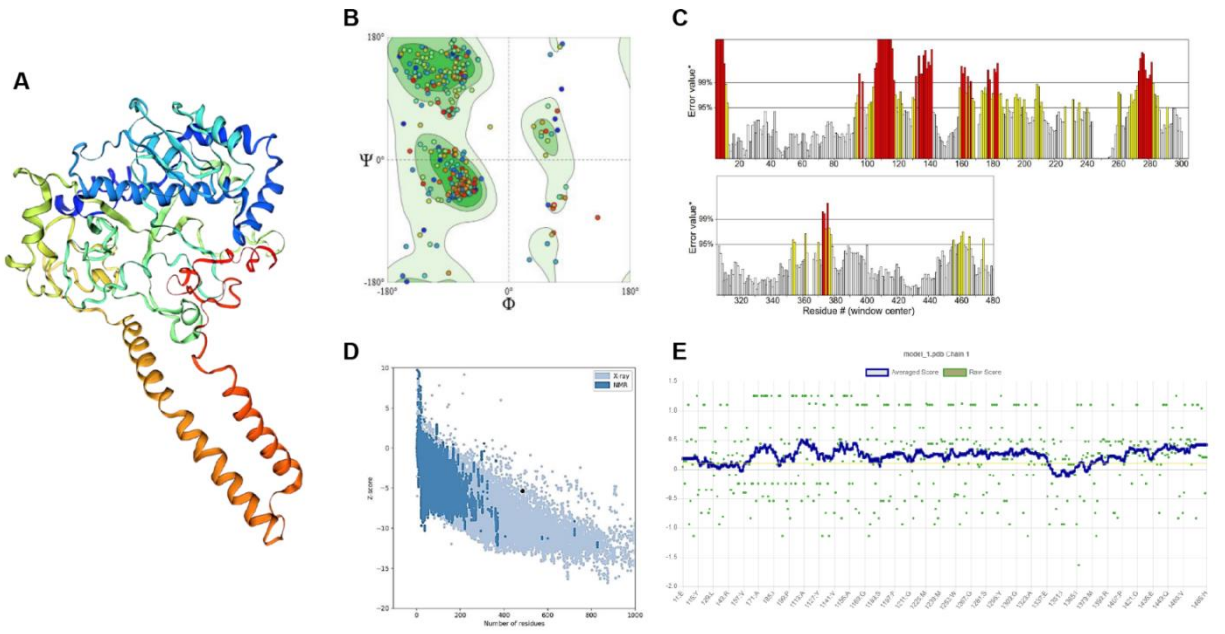

Fig. 3: Structure prediction and validation of vaccine construct: (A) The predicted 3D structure (B) Ramachandran plot analysis from SWISS-MODEL server (C) ERRAT analysis (D) Z-score analysis from ProSA-web and (E) Verify 3D analysis of the vaccine construct.

Table-IV: Discontinuous B-cell epitopes predicted by ElliPro.

Sl. no	Residues	No. of residues	Score
1	A:N396, A:H397, A:G398, A:V399, A:V400, A:K401, A:S402, A:M403, A:K404, A:K405, A:K406, A:P407, A:N408, A:T409, A:S410, A:E411, A:D412, A:L413, A:S414, A:K415, A:A416, A:E417, A:N418, A:E419, A:G420, A:G421, A:N422, A:D423, A:K424, A:K425, A:E426, A:D427, A:A428, A:A429, A:K430, A:P431, A:S432, A:S433, A:A434, A:E435, A:S436, A:K437, A:K438, A:K439, A:S440, A:V441, A:K442, A:T443, A:E444, A:G445, A:G446, A:R447, A:G448, A:Q449, A:H450, A:P451, A:D452	57	0.871
2	A:K348, A:K349, A:K350	3	0.85
3	A:I206, A:G207, A:K215, A:E216, A:S301, A:D302, A:I303, A:K304, A:S305, A:K306, A:G307, A:P308, A:G309, A:P310, A:G311, A:E312, A:K313, A:N314, A:K315, A:R317	20	0.732
4	A:E1, A:A2, A:A3, A:A4, A:K5, A:G6, A:I7, A:I8, A:N9, A:T10, A:L11, A:K13, A:Y14, A:Y15, A:C16, A:R17, A:V18, A:R19, A:G21, A:A24, A:Y91, A:D92, A:L93, A:D94, A:P95, A:A96, A:L98, A:V316, A:G318, A:K319, A:L320, A:T321, A:D322, A:A323, A:S324, A:K325, A:K326, A:G327, A:G328, A:E329, A:E330, A:F331, A:N332, A:K333, A:S334, A:K335, A:K336, A:E337, A:E338, A:Q339, A:I340, A:D343, A:P344, A:S345, A:L346, A:E347, A:I351, A:S352, A:I353, A:S354, A:K357, A:S358, A:K359, A:K360, A:R361, A:T362, A:K363, A:K364, A:I365, A:G366, A:I367, A:E368, A:G369, A:S370, A:R371, A:I372, A:K373, A:H374, A:E375, A:K376, A:K377, A:M379	82	0.639
5	A:L29, A:P30, A:K31, A:E32, A:Q34, A:I35, A:G36, A:K37, A:C38, A:S39, A:T40, A:R41, A:G42, A:R43, A:K44, A:C45, A:C46, A:R47, A:R48, A:K49, A:K50, A:E51, A:A52, A:A53, A:A54, A:K55, A:V57, A:Y68, A:I69, A:P99, A:F100, A:A101, A:I111, A:Y112, A:Y114, A:Q129, A:L130, A:G131, A:G132, A:V133, A:D134, A:A135, A:L136, A:A137, A:Y138, A:Y139, A:F140, A:V141, A:V142, A:G143, A:F148, A:A149, A:Y150, A:Y151, A:K152, A:Y153, A:I154, A:A155, A:S156, A:Y157, A:A158, A:A159, A:Q160, A:L161, A:G162, A:G163, A:V164, A:D165, A:A166, A:G167, A:P168, A:G169, A:P170, A:G171, A:D172, A:L173, A:I174, A:Q175, A:I257, A:F258, A:W259, A:D260, A:E261, A:A262, A:V263, A:N264, A:Q265, A:F266, A:G267, A:P268, A:G269, A:P270, A:G271, A:K272, A:S273, A:T274, A:E275, A:N276, A:T277, A:V278, A:Y279, A:L280, A:S281, A:V282, A:P283, A:S284, A:K285	107	0.628
6	A:D390, A:R393, A:K394	3	0.601

Molecular Docking Analysis & Binding Affinity Prediction of the Vaccine Construct: The docking process of the vaccine construct and human receptor TLR-4 (PDB ID: 4G8A) was performed using the ClusPro2.0 server, which generated 29 models for each complex. Among them, the model with the highest cluster size of 47 and the lowest energy score of -990.2 was selected for the vaccine-TLR-4 complex (Figure 4A). The docked poses with the highest cluster member were subsequently analyzed for binding affinities at 37°C utilizing the PRODIGY web server, which provided the ΔG values of the vaccine-TLR4 complex as - 8.1.

Molecular Dynamic Simulation Study of the Vaccine and TLR-4 complex: The molecular dynamic simulation of vaccine-TLR4 complex was performed by the iMODS server. The peaks in the deformability curve demonstrated flexible regions. There were few such peaks in vaccine-TLR4 complex which illustrates stable binding (Fig. 4B). Few fluctuations of atomic displacements were observed for the vaccine-TLR4 complex (Fig. 4C). The eigenvalue for the complex was 5.805177e-06 (Fig. 4D), and there is an inverse relationship between normal mode variance and the eigenvalues. The purple bars indicate variance of individual modes while the green bars indicate cumulative variance (Fig. 4E). The coupling between pairs of residues is illustrated by the covariance map where red, white and blue color corresponds to correlated, uncorrelated and anti-correlated motions (Fig. 4F). The elastic network shows the pairs of atoms connected by a spring. The darker the grey color, the stiffer the spring (i.e. more rigid positions in the complex) (Fig. 4G).

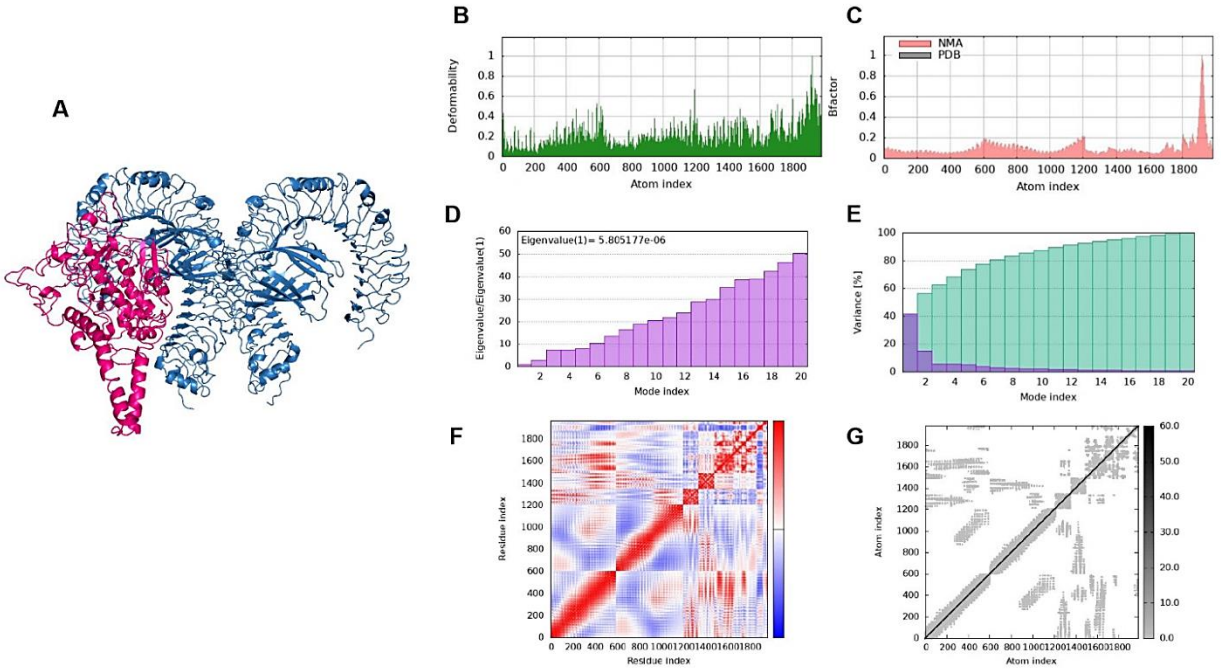

Fig. 4: Output of normal mode analysis (NMA) study by iMODS: (A) The vaccine-TLR4 complex, (B) Deformability plot, (C) B-factor plot, (D) Eigenvalue plot, (E) Normal mode variance plot. The purple bars indicate variance of individual modes while the green bars indicate cumulative variance (F) Covariance map. Red, white and blue color corresponds to correlated, uncorrelated, and anti-correlated motions (G) Elastic network. The darker the grey color, the stiffer the spring.

In silico Immune Simulation Studies: The simulated immunological response was investigated through the C-ImmSim server. The primary response was characterized by high IgM levels. B-cell populations and the levels of antibodies (IgG1+IgG2, IgM, and IgG+IgM) increased enormously after the second and third injections with concomitant reduction of the antigen levels (Fig. 5 A&B). The TH (T helper) and TC (T cytotoxic) cell populations responded remarkably with the corresponding memory development (Fig. 5 C&D). After the third injection, IgG1 levels increased, while IFN-γ concentration and TH cell population remained high throughout the exposure period. The level of cytokines after each injection increased concomitantly, reflected by the escalation of IFN-γ and IL-2 (Fig. 5E).

Codon Optimization and in silico Cloning: The amino acid sequence of vaccine was translated back to a cDNA nucleotide sequence to inspect the expression and to clone the constructed vaccine inside the pET-28a (+) vector. After codon optimization, the vaccine construct showed 48.465381% GC content, with the codon optimization index (CAI) value being 1.0, and the selected target organism was *Escherichia coli* (strain K12). Afterwards, SnapGene software (version 8.0.1) was used to integrate the adapted DNA sequence to pET-28a (+) vector, between the *Eco53kI* and *FspI* restriction sites (Figure 6). This vector enables enhanced protein recovery and purification due to its N-terminally 6×His-tagged proteins.

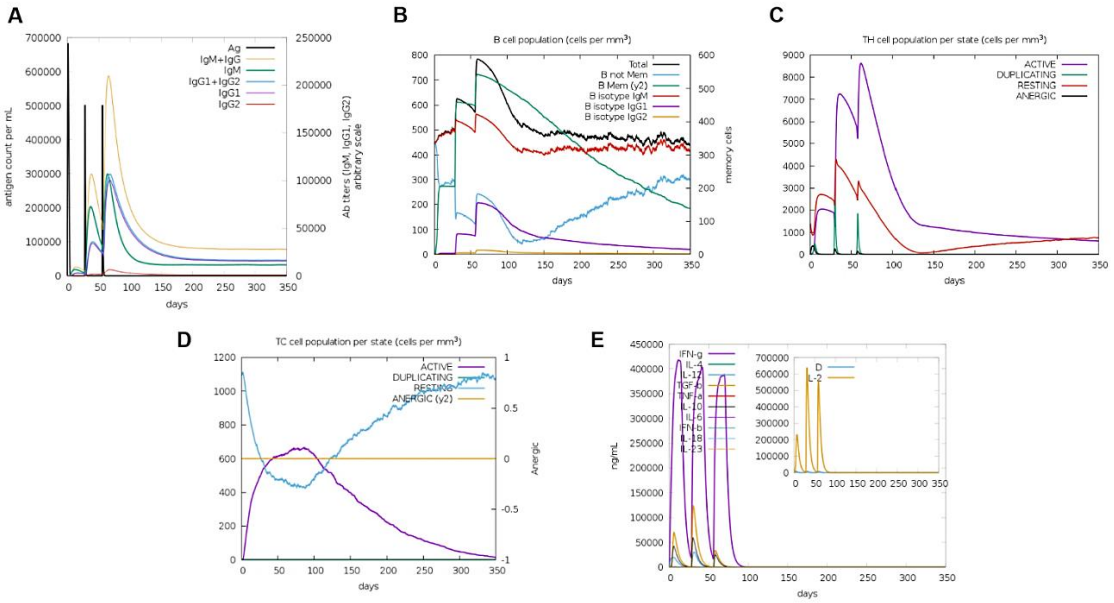

Fig. 5: Immune simulation response against vaccine construct: (A) Antigen and immunoglobulins, (B) B-cell population, (C) TH (helper) cell population (D) TC (cytotoxic) cell population per state, (E) Cytokine and interleukin production.

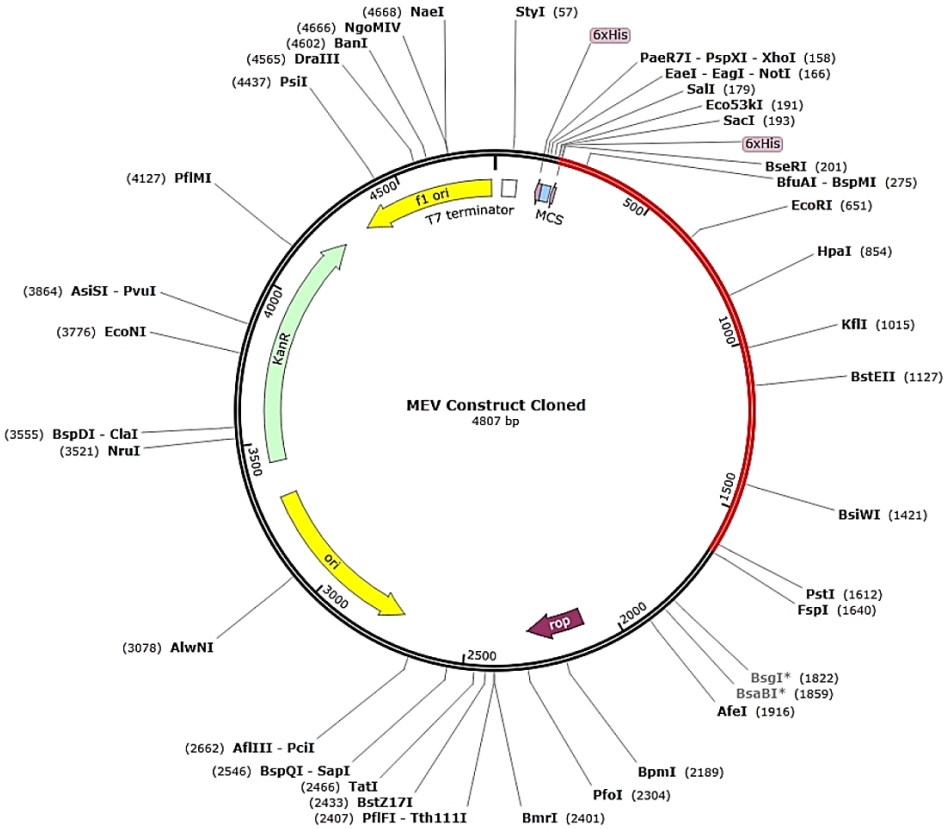

Fig. 6: *In silico* cloning of MEV gene in a restriction cloning vector pET28a (+) in *E. coli* host. Here, the red areas indicate the MEV, and the black areas represent the expression vector, pET28a (+).

Discussion

In order to combat the rising burden of periodontal diseases, new vaccine design is of extreme importance and a demanding scientific challenge.[8] The progression made in sequence-based technology and with blessings of computational studies, and the available genomics and proteomics data of different pathogens makes it possible to propose peptide-based vaccines. The reverse vaccinology approach has been proven to be a promising approach widely used against *Meningococcus B*, *Chlamydia pneumoniae*, *Salmonella typhii*, and *Legionella pneumophila*.[14,13,35,34] In current era, no vaccines are available against *T. denticola*, and therefore, the development of new vaccine model against periodontal pathogen is necessary. According to previous research[15], 11 essential nonhomologous target proteins were identified and characterized as potential drug targets from the entire proteome of *T. denticola* using the Subtractive Genomics approach. In the current study, we formulated the reverse vaccinology approach on these newly identified and characterized target proteins of *T. denticola* for vaccine target identification and vaccine modelling. Out of the 11 target proteins, two proteins (namely, Acetate kinase AckA and Chromosomal replication initiator protein DnaA) were chosen based on their high antigenicity scores by Vaxijen2.0 server (Table 1) and were selected for epitope prediction. Both the target proteins were cytoplasmic proteins, non-homologous to human proteins, essential for the survival of the pathogen, and were involved in important metabolic pathways of the pathogen (i.e, Acetate kinase AckA in pyruvate metabolism, propanoate metabolism, methane metabolism, taurine and hypotaurine metabolism, along with other carbon fixation pathways, whereas, Chromosomal replication initiator protein DnaA in two-component system and cell division pathways).[15]

Afterwards, we used the IEDB MHC-II prediction tool to predict the HTL (15mer) epitopes, NetCTL 1.2, IEDB Immunogenicity, and MHC-I prediction tools for CTL (9mer) epitopes, and the BepiPred2.0 server to predict LBL epitopes (Table 2). The CTL epitopes were further screened for their immunogenicity and binding scores, HTL epitopes for interferon-γ, IL-4 and IL-10 production, and LBL epitopes for transmembrane topology and IgG inducibility. All epitopes, afterwards, were analyzed for their antigenicity, toxicity, and allergenicity and the most antigenic, non-toxic, and non-allergenic epitopes were used for the construction of our multi-epitope vaccine, as reported in several other studies.[11,12,52] The selected epitopes containing 8 CTL, 8 HTL and 8 LBL epitopes were then fused together using AAY, GPGPG, and KK linkers, respectively (Figure 2). The GPGPG linker inhibits the formation of the 'junctional epitope' and aids in immune processing, while the AAY linker improves epitope immunogenicity by affecting peptide stability.[53] The KK linker improves the maintenance of independent immunogenic functions of the constructed vaccine.[53] The adjuvant, human beta-defensin-3 was added to the N terminal domain of the final multi-epitope vaccine using an EAAAK linker to increase immunogenicity, synthesizing the vaccine construct with 485 residues (Figure 2A and C). Consequently, the newly designed vaccine was predicted to be antigenic, non-toxic, and non-

allergenic by Vaxijen2.0, ToxinPred2, and AlgPred2 respectively. Physicochemical property analysis revealed that the vaccine was basic, stable, hydrophilic, and thermostable in nature (Table 3). The predicted secondary structure of the final vaccine by PSIPRED has been exhibited in the (Figure 2B).

The final vaccine construct was modelled using I-Tasser server, and because of the lack of any suitable template for homology modelling, this ab-initio approach was used. The structure was refined through the GalaxyWEB server. This server follows a refinement method that rebuilds the side-chains along with side-chain repacking. It also causes structural relaxation using molecular dynamics simulation, improving the structural quality significantly.[40] Following the refinement, the structure was validated through SAVESv6.1, ERRAT, ProSA-web, and SWISS-MODEL Structure Validation servers. The Ramachandran plot showed <85% residues allocated in the most favourable region validating the modelled vaccine tertiary structure is of high quality.

Moreover, the docking interaction of modelled vaccine construct with human TLR4 (PDB ID: 4G8A) was performed using ClusPro2.0. From the outcome, the vaccine-TLR4 complex that appeared to be docked with maximum residue interaction and lowest interaction energy was selected (Figure 4A), because the lower energy score corresponds to the better binding affinity. The docked poses with the highest cluster member were subsequently analyzed for binding affinities at 37 °C utilizing the PRODIGY web server, which provided the ΔG values of the vaccine-TLR4 complex. It was observed that all the interactions were energetically feasible, which is evident from the negative values of Gibbs free energy. Several studies highlighted the importance of interaction of vaccines with TLR4.[12,35,52]

Furthermore, the iMODS server was used for the prediction of the binding stability and flexibility of the vaccine-TLR4 complex. Deformability depicts the flexibility of the protein whereas B-factor is a measure of the mobility of the protein.[54] The relative amplitude of the displacements of atoms about an equilibrium position is indicated by B-factors.[48] The peaks in the deformability curve demonstrated flexible regions and very few peaks were observed in the deformability plot (Figure 4B) that illustrated high stability of the complex with an extremely low chance of deformability. The large eigenvalue of 5.805177e-06 (Figure 4D), showed that high energy is required to deform the complex. The complex had a high number of correlated residues as shown by the red color in the covariance map (Figure 4F). A higher number of stiffer regions was present in the vaccine-TLR4 complex, as illustrated by the grey dots in the elastic network (Figure 4G). The results from iMODs demonstrated stable binding of the vaccine-TLR4 complex, as reported in several other studies.[12,35,52]

Afterwards, *in silico* immune simulation was performed to evaluate the immunogenic potential of the vaccine. The results indicated that the main vaccination scheme, including 1, 84, and 168 time-steps had overall superior immunological responses. The results indicated a general increase in immune responses following repeated exposure to the antigen (Figure 5). B-cell and T-cell populations both responded remarkably with

corresponding memory development. The levels of IL-2 and IFN-γ were also increased, which are cardinal for immune system activation.

Additionally, because of the codon bias, codon adaptation is a vital step in reverse vaccinology which can promote recombinant protein expression significantly, and therefore, the codon was optimized for the vaccine construct by converting its sequence to cDNA using the JCat server to ensure the successful expression in the pET-28a (+) vector. The normal estimated GC content range is 40–60%, with the codon optimization index (CAI) value being more than 0.5. The study highlighted 48.465381% GC content and 1.0 CAI value, representing the efficacious expression of vaccine construct. The optimized vaccine sequence was then successfully cloned in the pET-28a (+) vector with *Eco53kI* and *FspI* as the restriction enzyme cleavage sites by using the SnapGene v8.0.1 tool (Figure 6). This vector enables enhanced protein recovery and purification due to its N-terminally 6 × His-tagged proteins.[52] Comparably, several studies highlighted this process but with the use of different restriction enzymes.[34,35,52]

The current study, up to our knowledge, is the first *in silico* based vaccinology study conducted against *T. denticola* (strain ATCC 35405) and we believe that it could provide an attractive alternative approach to tackle the dissemination of *T. denticola* related infections.

Conclusion

In the current study, we have chosen highly antigenic, non-allergic, and non-toxic epitopes and concatenated suitable linkers along with an adjuvant to design a multi-epitope-based vaccine against *T. denticola* utilizing various immune-informatics and bioinformatics tools. As compared to the traditional vaccine development methods, immunoinformatics is a more useful, easier, and faster process. The constructed multi-epitope vaccine appears safe to use and is capable of eliciting both innate and adaptive immunity (humoral and cellular). Nevertheless, further *in vitro* and animal studies are warranted to strengthen the efficacy of the designed multi-epitope vaccine for its possible utility as a potent preventive measure.

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