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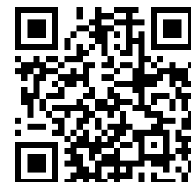
IMMUNOINFORMATICS-GUIDED SYNTHESIS OF A MULTI-EPITOPE VACCINE CANDIDATE ADVERSE TO UREAPLASMA UREALYTICUM TARGETING THE MULTIPLE BANDED ANTIGEN



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Abstract
Ureaplasma urealyticum is an opportunistic pathogen that is associated with numerous urogenital infections, infertility, and pregnancy complications. The lack of a cell wall and the growing resistance to traditional antibiotics lead to the need to develop alternative preventative measures like vaccines. A multi-epitope subunit vaccine against the MBA protein, which is a protein on the surface and is a virulence factor that interacts with the host immune system, and is also an antigenic variation protein, was conducted by a computational reverse vaccinology method. The MBA proteins were examined to determine possible B and T-cell antigens of proteins that had high antigenicity, non-allergenicity and immunogenic potential. The epitopes of interest were combined into a multi-epitope construct with a suitable adjuvant to boost immune response. Structural modelling, immune receptor molecular docking, and immune simulation research were executed to observe the stability and efficacy of the created vaccine candidate. These findings exhibited a high binding affinity with Toll-like receptor, and cellular immune responses and humoral elicitation. Moreover, in-silico cloning was done by using E. coli as an expression vector. Overall, this study presents a promising in-silico platform to develop an efficient vaccine against U. urealyticum as a possible step to control infections with this pathogen before other experimental data is collected.
Keywords: *Epitopes, Infertility, MBA protein, Structure prediction, Urogenital infections, Vaccine*

INTRODUCTION

Ureaplasma urealyticum is a bacterium that has a wall-less of class mollicutes, which is an opportunistic pathogen of clinical significance and members of which are highly dependent on the host for metabolism and have reduced genomes (1). The organism is usually found in the human urogenital tract and is often found in sexually active people (2). While asymptomatic colonization occurs, the role of *U. urealyticum* in non-gonococcal urethritis, infertility, pelvic inflammatory disease, adverse pregnancy outcome and neonatal complications is growing in importance (3, 4). *U. urealyticum* is known to have several virulence factors such as urease activity causing ammonia production, adherence to epithelial cells, antigenic variation, biofilm formation and induction of inflammatory response (5). One of the most important surface-exposed lipoproteins in the host-pathogen interaction and immune evasion is Multiple Banded Antigen (MBA), which is a virulence associated protein (6). The phase and size variation of the MBA protein enables the bacterium to escape the immune system of the host and to initiate persistent infections (7, 8). Additionally, TLR activation by administration of an mRNA also leads to the induction of pro-inflammatory cytokines that are involved in tissue damage and chronic inflammation in the urogenital tract (9).

The prevalence of *U. urealyticum* has significantly risen worldwide in the recent years, especially in women of reproductive age and pregnant women (10). Multiple epidemiological investigations have shown that the presence of *U. urealyticum* colonization is highly correlated with reproductive issues such as preterm birth, chorioamnionitis, spontaneous abortion, and neonatal respiratory diseases (11). In addition, other sexually transmitted infections (STIs) like *Chlamydia trachomatis* and *Mycoplasma hominis* may also exacerbate

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inflammatory responses and aggravate disease severity (12). Infections by *U. urealyticum* are underdiagnosed, particularly in developing countries, where molecular diagnostic capacities are still low (13). The use of antibiotics to treat infections caused by *U. urealyticum* has become more difficult due to intrinsic and acquired resistance (14, 15). As a result of the absence of peptidoglycan cell wall, the organism is naturally resistant to β-lactam antibiotics. In addition, resistance to macrolides, tetracyclines and fluoroquinolones has developed worldwide (16). Conventional antimicrobial treatments are less effective due to mutations in the genes encoding resistance determinants for ribosomes and quinolones and acquisition of such determinants like tet (M). This has led to treatment failures and chronic infections and highlighted the need for alternative preventive and therapeutic approaches (17, 18).

Vaccine synthesis is now revolutionised by reverse vaccinology approaches enabled by recent developments in computational biology and immunoinformatics (19). In contrast with traditional vaccine development approaches, reverse vaccinology relies on genomic and proteomic information to determine antigens (antigenic proteins) and immunogenic epitopes (immunogenic peptides), which are capable of inducing protective immune responses (20, 21). This approach is especially beneficial for pathogens that have few metabolic pathways, antigenic variation and complex host interactions, like *U. urealyticum* (22). The multi-epitope vaccine design approach is based on the idea of combining multiple T-cell and B-cell epitopes into a single vaccine construct, thereby boosting the immunogenicity of the vaccine and reducing the risk of adverse reactions commonly found in whole-cell vaccines (23).

The present study used an immunoinformatics-based reverse vaccinology method for designing a potential multi-epitope subunit vaccine against U. urealyticum. The surface accessibility, immunogenicity, and central role in pathogenesis of the MBA protein were the reasons for being chosen as the primary vaccine target. Antigenicity, toxicity, allergenicity and immunogenic potential were predicted for epitopes before they were added to a vaccine construct. In addition, structural modelling, molecular docking with immune receptors, immune simulations and in silico cloning analyses were performed to check the stability and efficacy of the proposed vaccine. The computational framework developed in this study could be used in the development of novel vaccine strategies against *U. urealyticum* and may help in controlling infections caused by this emerging pathogen.

METHODOLOGY

The methodology for developing a chimeric vaccine against *U. urealyticum* is divided into three sequential phases: prediction, construction, and analysis (Fig. 1). The initial prediction phase begins with retrieving the Fasta sequence of target proteins, followed by protein analysis, epitope prediction, and subsequent epitope analysis. In the construction phase, the identified epitopes are used for vaccine designing, followed by structure prediction, molecular docking, and Normal Mode Analysis (NMA) evaluation to assess stability and interactions. Finally, the analysis phase evaluates the vaccine's efficacy through population coverage prediction, conformational B-lymphocyte (CBLs) prediction, immune simulation, and in-silico cloning to confirm expression potential. This workflow ensures a comprehensive computational approach for vaccine design.

PROTEIN CANDIDATE ANALYSIS

The sequences of all vaccine MBA proteins in the genome of *U. urealyticum* were taken from NCBI database under accession numbers WP_004025498.1, WP_004025600.1, WP_004026047.1, WP_004026079, WP_004026130.1, and WP_230438566.1.1, respectively. All of the proteins were exposed to physicochemical parameter analysis by ExPASy ProtParam tool [27]. The antigenic property was detected using VaxiJen version 2.0 server (1), while the demonstrates allergenic properties using the AllerTOP version 2.0 server (2). Furthermore, the non-toxicity of the proteins was confirmed via the CSM-Toxin server (3), and the Innovagen server gave the water solubility property of these proteins (4).

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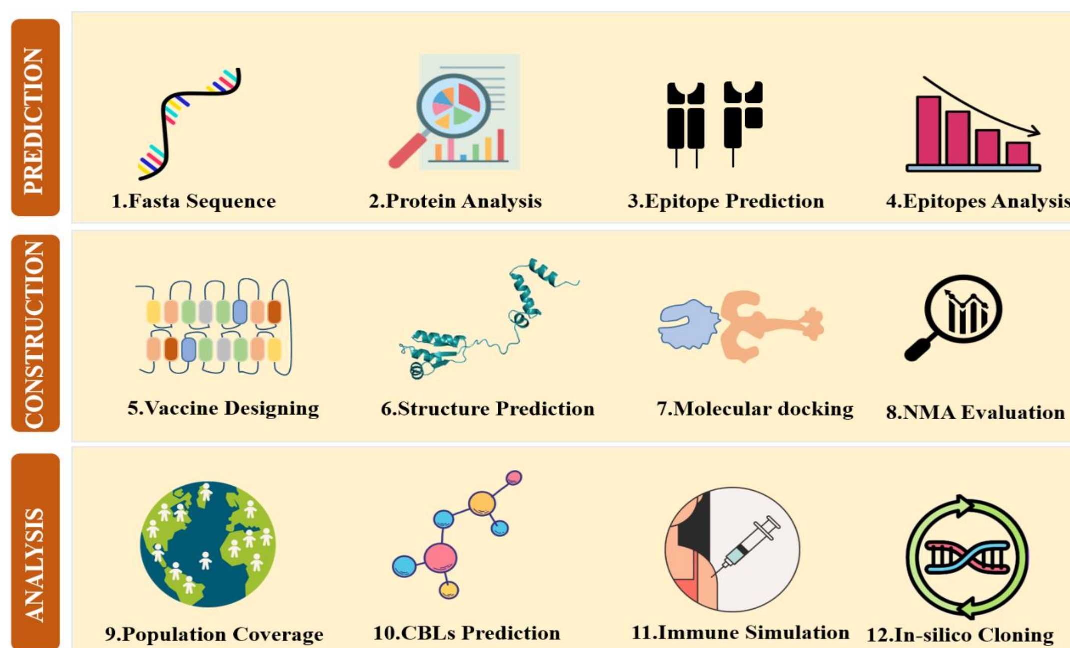

Fig. 1. Schematic workflow of the computational pipeline used for the rational design of a chimeric vaccine against *U. urealyticum*

T-CELL PREDICTION AND ANALYSIS

Predictions for MHC-I and MHC-II epitopes were performed by NetMHCpan 4.1EL [71] by employing a set of 27 common HLA alleles in reference, through IEDB MHC-I (http://tools.iedb.org/mhci/) and MHC-II (http://tools.iedb.org/mhcii/) web servers. For the screening of MHC-I epitopes, cutoffs of ≥ 0.9 for prediction score and ≤ 0.05 for percentile rank were used. For MHC-II epitopes, same cut-off scores were applied. Finally, selected epitopes were subjected to VaxiJen 3.0 to validate their antigenecity, AllerTOP v2.1 to test allergenicity, and CSM-Toxin to screen toxicity. Moreover, the innovagen server detected the water soluble properties of these epitopes.

B-CELL PREDICTION AND ANALYSIS

B-cell receptors are globular proteins that are anchored on the cell membranes and allow B-cells to identify the antigens existing in their soluble forms. Once activated, B-cells release antibodies, that are the immunoglobulins soluble form. Antibodies mediate adaptive humoral immunity. Interaction between the antibodies and target molecules results in various consequences. BCEPREDS server predicted these epitopes from protein sequences (5). Later, the epitopes were subjected for non-allergenic, antigenic, soluble, and non-toxic properties.

VACCINE DEVELOPMENT

T-cell and B epitopes with ability to stimulate an immune response were retrieved and combined with the adjuvant to form a multi-epitope vaccine. Adjuvant selected was 50S L7/L12 ribosomal protein adjuvant. The vaccine was designed such that adjuvant was connected to the N-terminal end. The linkers chosen for this task include EAAAK, GPGPG, and AAY. The vaccine design involves the combination of adjuvant, MHC-I, MHC-II, and B cell epitopes through EAAAK, GPGPG, AAY, and KK linkers**.** EAAAK were subjected at N and C-terminal to give rigidity, while AAY was to join MHC-I, GPGPG for MHC-II and KK for joining B-cells (6).

SECONDARY STRUCTURE PREDICTION

The sequence designed was further subjected for secondary structure prediction to detect the helices, loops, strands and sheets. For this purpose the SOPMA (7) cand PSIPRED server (8) were utilized to predict secondary structure and its properties. Number of residues of Alpha helices, Beta-sheets, random

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coils, and loops in 2D and their overall ratios were predicted via SOPMA. On the other hand, the visualization was done by PSIPRED.

TERTIARY STRUCTURE VALIDATION AND PREDICTION

3D models for multi-epitope vaccine complex have been predicted through AlphaFold server (9). The GalaxyRefine web server has been employed to optimize the predicted vaccine models (10). Through the ERRAT, and PROCHECK web servers, the accuracy of 3D structure of optimized vaccine model has been further assessed (11).

MOLECULAR DOCKING

Docking analysis of interaction of the vaccine (refined final model) with Toll-like receptor 4 (TLR4) (PDB ID: 4G8A) was done using ClusPro 2.0 (http://www.cluspro.org/help.php) server (12), which uses PIPER algorithm according to the default conditions. The structure models of toll-like receptor 4 was acquired using RCSB in the form of pdb files. Docking complexes with the least energy score and docking efficiency were identified from the outcome obtained. Moreover, the best docking complex was subjected to determination of the hydrogen bond interaction. Visual analysis of the interactions within the docked complex was carried out by means of PyMOL tool.

NMA EVALUATION

MD simulations have also been carried out through iMODS (https://imods.iqfr.csic.es/) (13). This tool performs NMA on docked complexes and evaluates their dynamics and stability. The best docked model of Vaccine-TLR5 complex obtained from the Cluspro server has been used for input in PDB format. Prior to submission to iMODS, the structural integrity and atom numbering have been checked in the input structure. The results from iMODS analysis, which include eigenvalue graphs, deformability graphs, B-factor distribution, variance graphs, covariance matrix, and elastic network maps, all contributed to the validation of dynamic stability of these docked complex 10.1038/s41598-026-39790-z.

POPULATION COVERGAE

Population coverage for chosen epitopes was analyzed using IEDB Population Coverage Tool (https://tools.iedb.org/population/). The potential to recognize epitopes that are capable of binding to HLA molecules was estimated using population coverage tools for the whole world and particular regions. Frequencies of HLA class I and II allele occurrence in different ethnic groups was taken into account. Population coverages both as a whole were calculated.

IMMUNE SIMULATION

Computational immune simulations were performed using the C-ImmSim online simulation platform (https:// kraken.iac.rm.cn. rncn. it/C-IMMSIM/). The simulation was run for 1000 time steps, equivalent to approximately one year, taking into account the need for memory formation in immune simulations. Three doses of MEV were administered during time steps 1, 84, and 168 (corresponding to days 0, 28, and 56). This schedule follows typical C-ImmSim prime-boost regimens for assessing Th1-polarized vaccines. The interval of 28 days between each dose is in accordance with the protocol proposed by Castiglione et al. (14) Cytokines and antibodies generated through C-ImmSim, characterized by elevated levels of IFN-γ and IL-2, indicative of a Th1-biased response, can be examined to evaluate the immunogenicity of the vaccine against *U. urealyticum* (15).

CBLS PREDICTION

Confirmational B-cell epitopes were projected by ElliPro server (http://tools.iedb.org/ellipro/) (16). ElliPro detects accessible epitopes that are clustered within the surface of proteins by employing the combination of geometric analysis of ellipsoid models of proteins with residue clustering methods. The method provides the possibility to predict conformational epitopes from vaccine constructs according to their three-dimensional structures for further immunogenicity studies (17).

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CODON ADAPTATION AND CLONING

Codon optimization was carried out to assess translational potential in *E.coli*. Codon optimization was done using JCat to optimize the vaccine construct for translation in E.coli K12. JCat software does codon optimization by replacing rare codons with codons which have similar frequency of occurrence to the codons in the selected host without any change in the primary sequence of protein amino acid. Optimization settings were designed such as to avoid rho independent termination signals, ribosome binding sequences. Later, optimized sequence was used in Snapgene software for in-silico cloning into Pet28(+) vector (18).

RESULTS

PROTEIN CANDIDATE ANALYSIS

All six proteins predicted to be membrane proteins were analyzed to identify a potential vaccine candidate. Only one protein WP_004025498.1 was predicted to be a potent candidate for vaccine designating. The immunogeneicty predicted by the VaxiJen v3.0 software was 100%, which make the protein best immune target, thus making the protein antigenic. Non-allergenicity of protein was confirmed by the AllerTOP v2.1 prediction server. Furthermore, the ProtParam server gives the profile of the protein, which possesses thermostable, alkaline, and hydrophilic nature, which implies its utility in the field of immunochemistry (Table I). Thermostable and alkaline score determine the stability, whereas hydrophilicity determines solubility in water and epitopes' availability. This protein was immunogenic, non-allergen, non-toxin, soluble, and got suitable physicochemical properties to be a vaccine candidate, so was subjected for epitope prediction.

Table I. Immunogenicity, allergenicity, toxicity, solubility and physicochemical analysis of selected MBA protein

Parameters	Protein	
	Measurement	**Indication**
Molecular Weight	84640.86	Appropriate
Number of Amino acids	267	Appropriate
Solubility	Good	Soluble
Immunogenecity	100%	Immunogen
Aliphatic Index	71.08	Thermostable
Instability Index	28.25	Stable
Allergenicity	Non-Allergen	Non-allergenic
GRAVY Value	-0.948	Hydrophilic
Theoretical Pi	6.87	Acidic

T-CELL PREDICTION AND ANALYSIS

CYTOTOXIC T-CELL PREDICTION

Using the 9-mer of the CTL with the available restriction alles set of 27 alleles, the epitopes were predicted using IEDB server, taking into consideration the IEDB-assisted criteria. Predicted epitopes were analyzed regarding their immunogenic, antigenic, allergenic, and toxic characteristics (Table1). In the process of immunological screening, various promising epitopes were found in of the target proteins; however, out of the promising epitopes found in the target proteins, one epitope with a high antigen score and neither allergic nor toxic nature was chosen from the CTL and HTL epitopes, respectively, and used to design the vaccine. Two CTL and were selected for to be incorporated into vaccine construct (Table II).

Table II. Analysis of non-overlpping CTL epitopes

Epitopes	Allele	Immunogen	Toxin	Allergen	Water solubility
AVVSSVEFK	HLA-A*11:01	66	No	No	Good
EASKTLSGK	HLA-A*68:01	100	No	No	Good

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HELPER T-CELL PREDICTION

Using the 15-mer of the HTL with the available restriction alles set of 27 alleles, the epitopes were predicted using IEDB server, taking into consideration the IEDB-assisted criteria. Top non-overlapping epitopes were selected to confirm their antigenicity, non-allergenicity, non-toxicity, and water solubility (Tables 3). In the process of immunological screening, many epitopes were found in of the target proteins; however, out of these epitopes found in the target proteins, only two epitopes with a high antigen score and neither allergic nor toxic nature was chosen from the HTL epitopes, respectively, and used to design the vaccine. Seven HTL were selected for to be incorporated into vaccine construct (Table III).

Table III. Analysis of HTL epitopes

S#	Epitope	Allele	Immunogen	Allergen	Toxin	Water solubility
1	SGKLSNLKEGTYNLS	HLA-DRB1*12:01	100%	No	No	Good
2	NQKTKQQAVDSAKEE	HLA-DRB4*01:01	100%	No	No	Good
3	ENQKTKQQAVDSAKE	HLA-DRB4*01:01	100%	No	No	Good
4	AEKELTTAKSDVETK	HLA-DQA1*03:01	100%	No	No	Good
5	QPSNLQKDKEEKVKA	HLA-DRB3*01:01	100%	No	No	Good
6	QKTKQQAVDSAKEEL	HLA-DRB4*01:01	100%	No	No	Good
7	KENQKTKQQAVDSAK	HLA-DRB4*01:01	100%	No	No	Good

B-CELL PREDICTION AND ANALYSIS

BCEPRED server available at IEDB server was utilized to predict the linear B-cell epitopes out of the selected protein. The epitopes were subjected to antigenic, soluble, non-allergenic and non-toxic properties. Out of all predicted epitopes, four LBL was selected due to its immunogenic, non-allergenic, non-toxic, and water soluble properties. Table IV shows the B-cell epitopes which were subjected further to be incorporated into vaccine construct.

Table IV. Linear B-cell epitopes analysis

S#	Epitopes	Score	Immunogen	Allergen	Toxin	Soluble
1	NTPTPSPTPTPKKDEAVVSS	1	100%	No	No	Good
2	TKKGETAPIQASDLKYDEAS	0.985	66%	No	No	Good
3	GPINEQSAQPSNLQKDKEEK	0.973	100%	No	No	Good
4	AKENQKTKQQAVDSAKEELK	0.931	100%	No	No	Good

VACCINE DEVELOPMENT

Based on the aforementioned epitope selection, a multi-epitope vaccine for *U. urealyticum*have been designed. In this construct, the adjuvants; L7/L12 Ribosomal protein adjuvant was used as the amino terminal part followed by a series of epitopes obtained from the that protein. This includes two MHC-I epitopes (in lavender), five MHC-II epitopes (in blue), and four B-cell epitopes (in salmon). Between adjuvant and first epitope, the linkers such as EAAAK (Pink) was employed, AAY (purple) were used between MHC-I epitopes while GPGPG (light blue) were inserted between MHC-II and KK for B-cell epitopes (Fig. 2). At last, again EAAAK was employed to secure the ends. Such a designed construct was expected to be safe and effective in in-silico vaccine development against *U. urealyticum*.

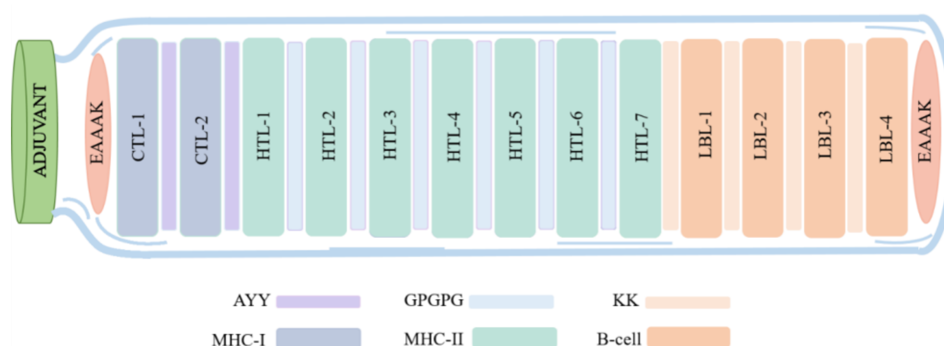

Fig. 2. A sequential representation of constructed vaccine via adjuvants and linkers

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The antigencity, allergenicity, toxicity, solubility and physichochemical analysis of the vaccine was predicted in order to justify it as immunogenic and potential vaccine against U. urealyticum. Our vaccine was predicted to be immunogenic 100%, non-allergen, Non-toxin, and water soluble. Moreover, the protparam server results concluded that vaccine was highly stable, and thermostable. Also, vaccine got appropriate length of amino achids, theoretical PI, and molecular weight. Negative GRAVY values further justified the hydrophilicity of the vaccine. Table V shows the detailed analysis done for designed vaccine.

Table V. Immunogenicity, toxicity and physicochemical analysis of vaccine

Parameters	V1	
	Measurement	**Indication**
Molecular Weight	39311.16	Appropriate
Number of Amino acids	380	Appropriate
Solubility	Good	Soluble
Immunogenecity	100%	Immunogen
Aliphatic Index	63.05	Thermostable
Instability Index	31.3	Stable
Allergenicity	Non-Allergen	Non-allergenic
GRAVY Value	-0.832	Hydrophilic
Theoretical Pi	8.52	Basic

MODELING OF 2D

Secondary structure prediction of engineered peptide vaccine was made using two online servers, namely PSIPRED and SOPMA, taking into account default values. The secondary structure of this engineered Vaccine was found to have an Alpha helix (41.58%) in 158 residues, Random coil (36.33%) in 93 residues, extended strand (7.03%) in 18 residues. Other characteristics related to the structural elements and their representation graphically can be seen in Fig. 3.

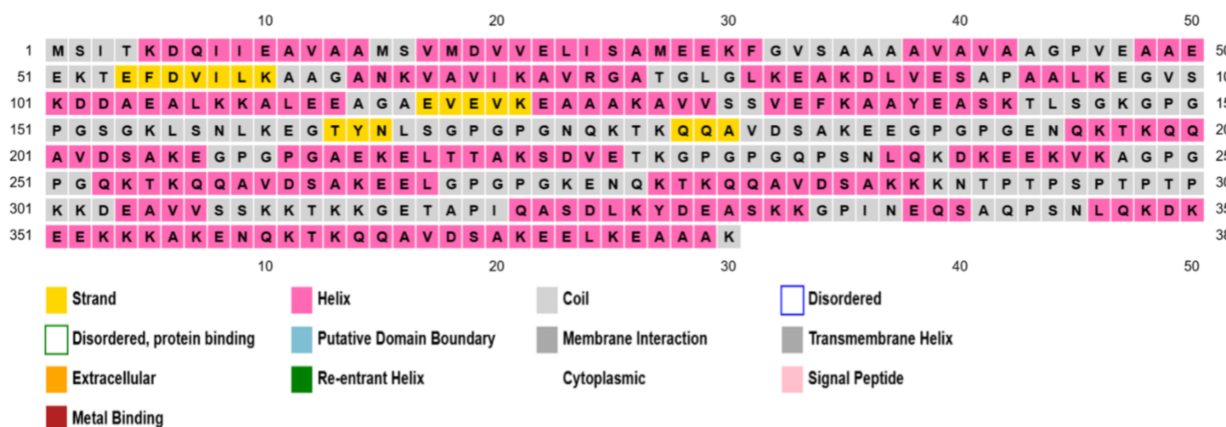

Fig. 1. PSIRPED server predicted vaccine secondary structure

TERTIARY STRUCTURE MODELING AND ANALYSIS

Created structural models of the designed chimeric vaccine obtained in three dimensions and the first one seemed to be most stable and clearly defined (Fig. 4a). This structure has been selected for further investigations. Alpha helices, Beta sheets, and loops can be seen in Figure 4A. Further, the selected model has been validated by ERRAT, and Ramachandran plot (Fig. 4b). The overall quality factor according to ERRAT analysis is equal to 93.373% (Fig. 4c). Analysis of the Ramachandran plot showed that more than 94.7% of amino acids are in favorable zones, whereas the remaining part is in the allowed and disallowed region (3.1% and 1.6%) (Fig. 4b). All of the mentioned above proves that the quality of selected model is sufficient for further modeling studies.

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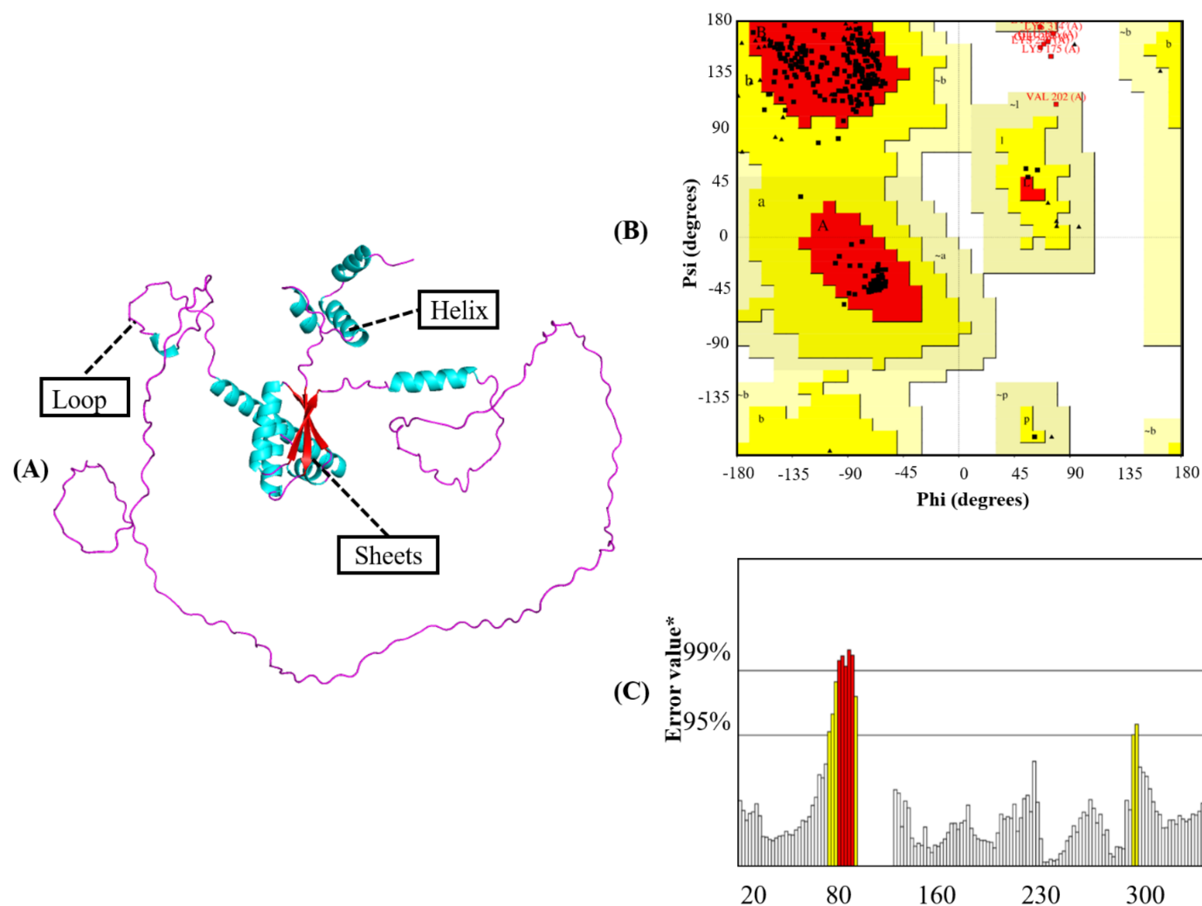

Fig. 2. Modeling and validation of PDB structure. **(a)** 3D of vaccine; **(b)** Ramachandran plot; **(c)** ERRAT plot

MOLECULAR DOCKING

Molecular docking was performed to check binding affinity and interactions made by vaccine with the host receptor. So, the human immune cell receptor like Toll-like receptor weas download from RCSB PDB in PDB format. TLR-4 was selected to be docked with the designed vaccine. Cluspro server predicted the interactions and binding affinity made by TLR4 and vaccine (Fig. 5a). The top complex with low energy was downloaded to be checked in PyMOL for measuring the number of interaction. The docking score made between Vaccine-TLR4 was -1173.7, while the hydrogen bond interaction calculated were 27 Fig. 5b.

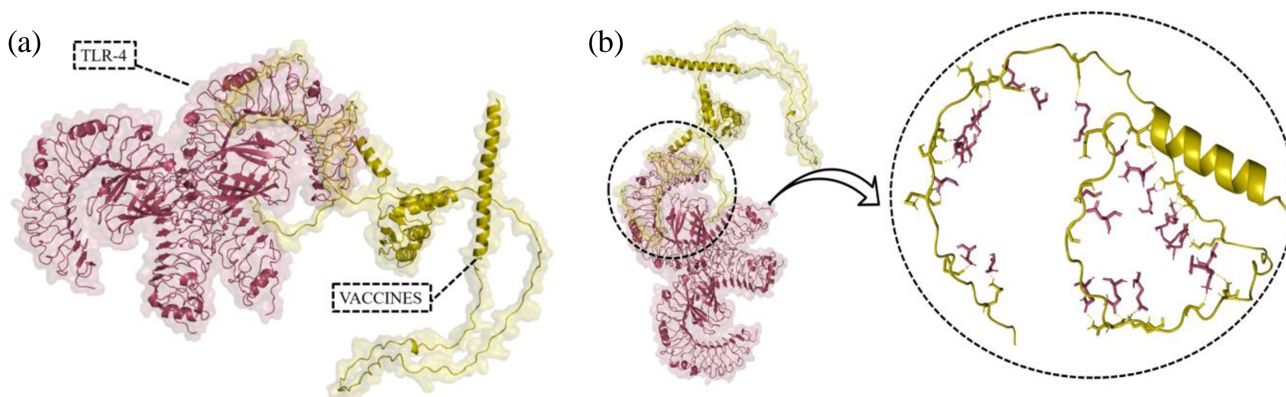

Fig. 3. (a) The docked complex of Vaccine and TLR4 downloded from CLuspro server; **(b)** The visualization of the interaction made among the vaccine and its docked receptor TLR4 via PyMOL

NMA EVALUATION

The simulation result obtained with iMODS reveals that the vaccine-receptor complex has less distortion in the vaccine structure and higher conformational stability, which suggests that the vaccine construct is well configured with immune receptor and has excellent structural dynamics in vaccine-receptor complex (Fig. 6). Motion and flexibility of molecules in antigen and receptor interface were

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analyzed using molecular simulations. The deformation curve for the Vaccine-TLR4 complex is shown in Fig. 6A, showing that a specific peak is observed in an area of flexibility in the vaccine structure. The intrinsic mobility of each residue is compared to the PDB and NMA structures in the B-factor analysis displayed in Fig. 6B. The eigenvalue (Fig. 6C) corresponds to the rigidity of the structure - the smaller the eigenvalue the more elastic the structure. The predicted e-value in present study was 1.057398e-08. The individual variance and total variance of the motions are shown in (Fig. 6D) in green and purple. The covariance matrix of residue pairs (Fig. 6E) depicts the correlation of each atom's motion. In the diagram, blue, red and white indicate correlated, anti-correlated and non-correlated motions respectively. An elastic network model is shown in Fig. 6F, which is similar to the spring map and shows the energy of interatomic interactions within the complex.

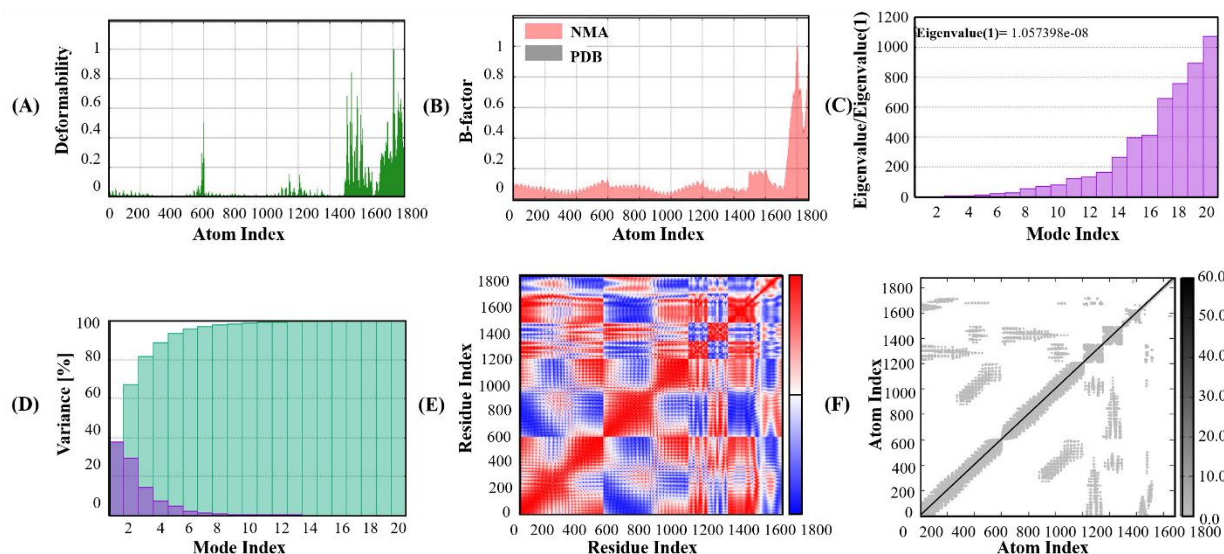

Fig. 6. Normal mode analysis evaluation of vaccine-TLR4 docked complex, (A) Deformability; (B) B-factor; (C) Eigen-value; (D) Variance; (E) Covariance; (F) Elastic network

COVERAGE OF PROTEIN ON GLOBAL SCALE

The IEDB Population Coverage tool was used to perform a population coverage analysis. The purpose of this analysis was to determine the most representative epitopes by the multiple ways of immune recognition. Use of curated data from IEDB database: Regional differences in the distribution of HLA and MHC alleles, which exist throughout the world, were considered. The regional variation is the result of complex interactions between environment and genetics. Population coverage is one of the key factors in making any vaccine effective in general. Using IEDB database, MHC-I and MHC-II population coverage was estimated in overall world was 98.61, securing most in Europe (99.70%). Fig. 7 shows a world map representing the results of population coverage analysis.

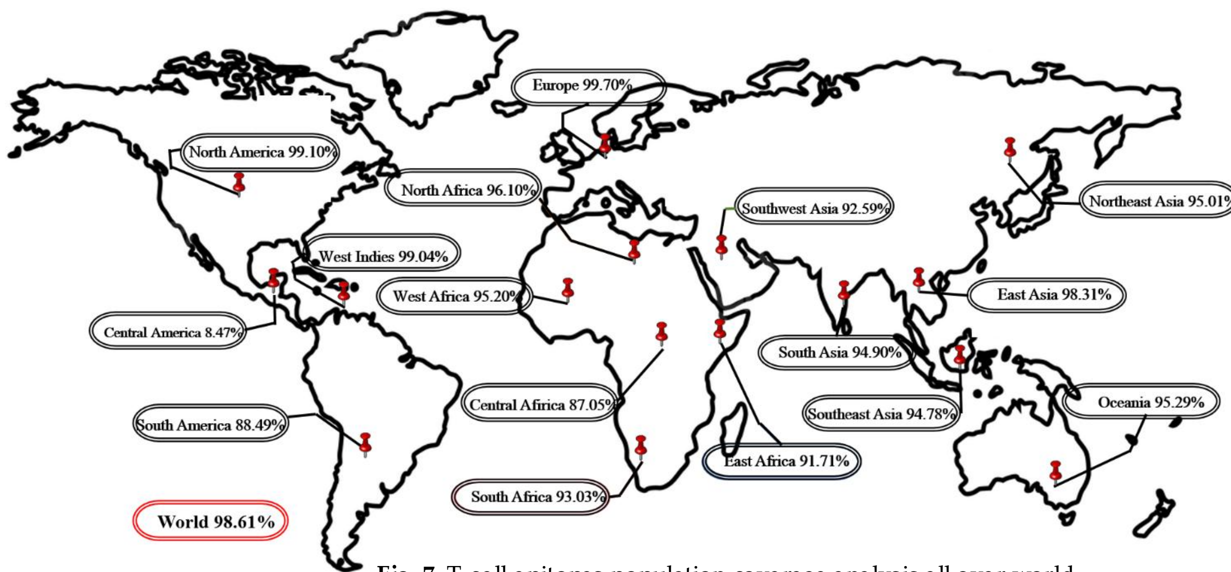

Fig. 7. T-cell epitopes population coverage analysis all over world

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IMMUNE SIMULATION

Prediction of efficacy of the vaccine response was done using C-ImmSim. Vaccine construct shows effective immune response in all cases of immune response from primary, secondary to tertiary immunity. From Fig. 8A, the type of antibody generation is highest for IgG and IgM, then IgM, IgG1, IgG1 + IgG2 and IgG2, respectively in secondary and tertiary exposures. In the case of Fig. 8B, the concentration of the cytokine and interleukins increases the most during first 50 days. IFN-γ and IL-2 showed very high concentrations, showing that the vaccine has been able to evoke a good immune response. In Fig. 8C, the number of active B cells is highly stimulated by the immune response. Likewise, in Fig. 8D and E, there is also an increase in population of helper T and cytotoxic T lymphocytes, but the population of cytotoxic T cells continues to increase.

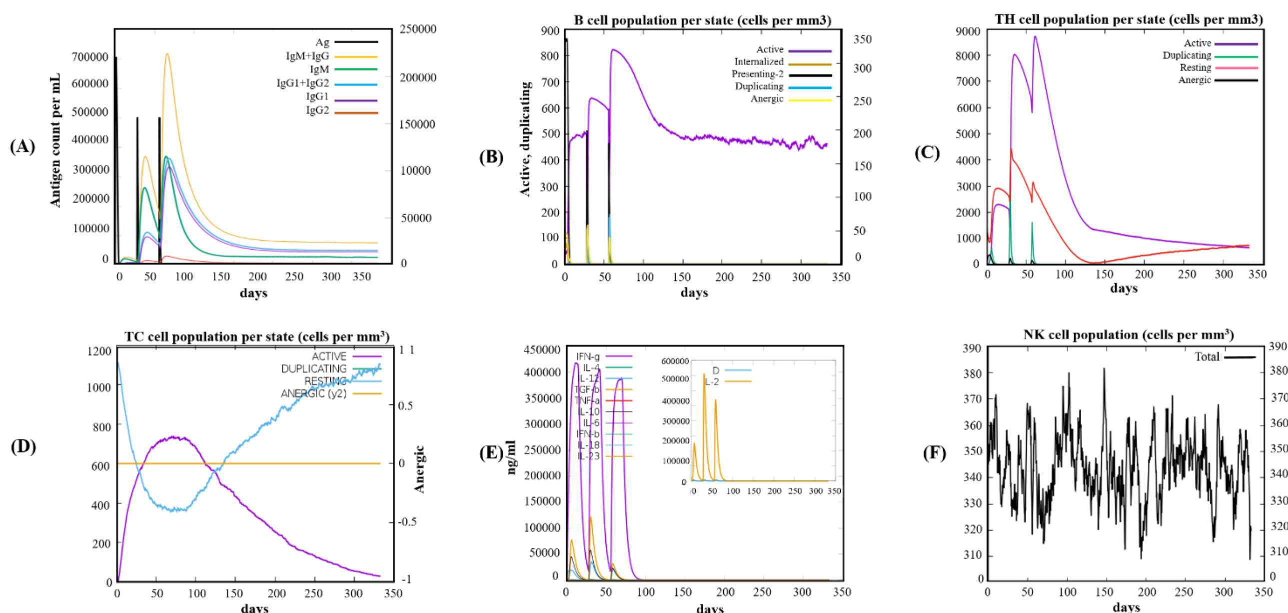

Fig. 8. C-immcim server predicted the immune simulation of designed vaccine. **(A)** Antibodies population; **(B)** B-cell population; **(C)** Helper T-cell Population; **(D)** Cytotoxin T-cell population 9; **(E)** Interleukins population; **(F)** Natural killer cell production

CBLS PREDICTION

Thirteen B cell epitopes having 189 residues were forecast by utilizing ElliPro server in Vaccine strucutre. Epitope 1 received the maximum prediction score of 0.887, signifying a highly accessible surface with the ability to elicit antibodies, while epitope the 13th had the minimum score of 0.549. All other epitopes scored acceptably well, suggesting their potential in eliciting antibodies against *U. urealyticum* (Fig. 9 and Table VI).

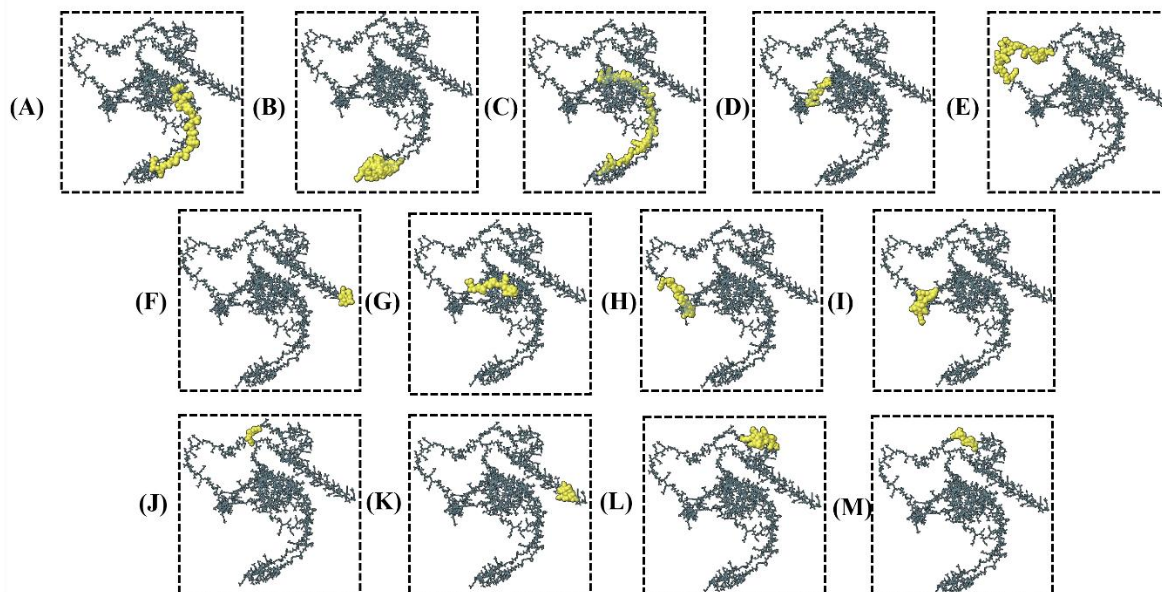

Fig. 4. The Elipro server predicted thirteen CBLs in the PDB structure of

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Table VI. Elipro server predicted 13 CBLs in the vaccine structure, with highest score of 0.887

No.	Residues	Number of residues	Score
1	A:K196, A:T197, A:K198, A:Q199, A:Q200, A:A201, A:V202, A:D203, A:S204, A:A205, A:K206, A:E207, A:G208, A:P209, A:G210, A:P211, A:G212, A:A213, A:E214, A:K215	20	0.887
2	A:E216, A:L217, A:T218, A:T219, A:A220, A:K221, A:S222, A:D223, A:V224, A:E225, A:T226, A:K227, A:G228, A:P229, A:G230, A:P231, A:G232, A:Q233, A:P234, A:S235, A:N236, A:L237, A:Q238, A:K239, A:D240, A:K241	26	0.822
3	A:E243, A:K244, A:V245, A:K246, A:A247, A:G248, A:P249, A:G250, A:P251, A:G252, A:Q253, A:K254, A:T255, A:K256, A:Q257, A:Q258, A:A259, A:V260, A:D261, A:S262, A:A263, A:K264, A:E266, A:L267, A:G268, A:P269, A:G270, A:P271, A:G272, A:K273, A:E274, A:N275, A:Q276, A:K277, A:T278, A:K279	36	0.781
4	A:Q280, A:Q281, A:A282, A:V283, A:D284, A:S285, A:A286, A:K287	8	0.779
5	A:K302, A:D303, A:E304, A:A305, A:V306, A:V307, A:S308, A:S309, A:K310, A:K311, A:T312, A:K313, A:K314, A:G315, A:E316, A:T317, A:A318, A:P319, A:I320, A:Q321, A:S323, A:D324, A:L325	23	0.769
6	A:E376, A:A377, A:A378, A:A379, A:K380	5	0.751
7	A:E187, A:G188, A:P189, A:G190, A:P191, A:G192, A:E193, A:N194, A:Q195	9	0.734
8	A:K288, A:K289, A:N290, A:T291, A:P292, A:T293, A:P294, A:S295, A:P296, A:T297, A:P298, A:T299, A:P300, A:K301	14	0.721
9	A:Q174, A:K175, A:T176, A:K177, A:Q178, A:Q179, A:A180, A:V181, A:D182, A:S183, A:A184, A:K185, A:E186	13	0.634
10	A:E329, A:A330, A:S331, A:K332	4	0.587
11	A:D368, A:S369, A:A370, A:K371, A:E372, A:E373, A:L374, A:K375	8	0.576
12	A:M1, A:S2, A:I3, A:T4, A:K5, A:D6, A:Q7, A:I8, A:I9, A:E10, A:A11, A:V12, A:A13, A:A14, A:M15, A:S16	16	0.559
13	A:K333, A:G334, A:P335, A:I336, A:N337, A:E338, A:Q339	7	0.549

CODON ADAPTATION AND CLONING

The chimeric multi-epitope subunit vaccine for E. coli K12 was codon optimized and the expression potential was improved. The optimized gene sequence had a relatively high CAI value as well as a balanced GC content, implying that it is highly transcribed and translated efficiently in the chosen host system. The best gene sequence obtained showed a CAI value of 1.0 and a GC content of 47.10% which were suitable for being expressed in the E. coli host system. The cloning approach was then simulated using the software SnapGene v3.3.4. The end of the gene sequence was modified to include restriction sites: EcoRV and FsPI to help the sequence insertion in the pET-28a(+) vector. No restriction sites were found within the sequence that would interfere with the cloning procedure. The optimized gene sequence was successfully cloned in the vector as shown in Fig. 10. The resulting clone was 5877bp long.

DISCUSSION

This species, *Ureaplasma urealyticum*, is one of the most paradoxical and biologically intriguing microorganisms of the class Mollicutes, with an extreme genomic simplicity and great adaptability (19). It lacks a cell wall, making it innately resistant to β-lactam antibiotics, and also increasing its resistance to treatment and persistence in clinical environments. One of the distinguished biochemical characteristics of *U. urealyticum* is its ability to catalyse the conversion of urea to carbon dioxide and ammonia, thus enabling production of ATP (20, 21). Additionally, the ammonia production is a factor in local pH modulation that may affect integrity of host tissue and immune responses. Also its mucosal tropism, due to the presence of multiple surface associated proteins, including multiple banded antigens (MBA), facilitates its adherence, colonization and immune evasion.

Although *U. urealyticum* is a well-known commensal in most healthy people, it is a controversial organism, lying on the edge between commensalism and pathogenicity (22, 23). Increased clinical evidence

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has linked it to a wide range of urogenital and obstetric complications such as chorioamnionitis, decreased fertility, and low-birth weight and preterm birth. Therefore, alternative therapeutic strategies are needed and vaccine development is seen as a viable and viable solution (24).

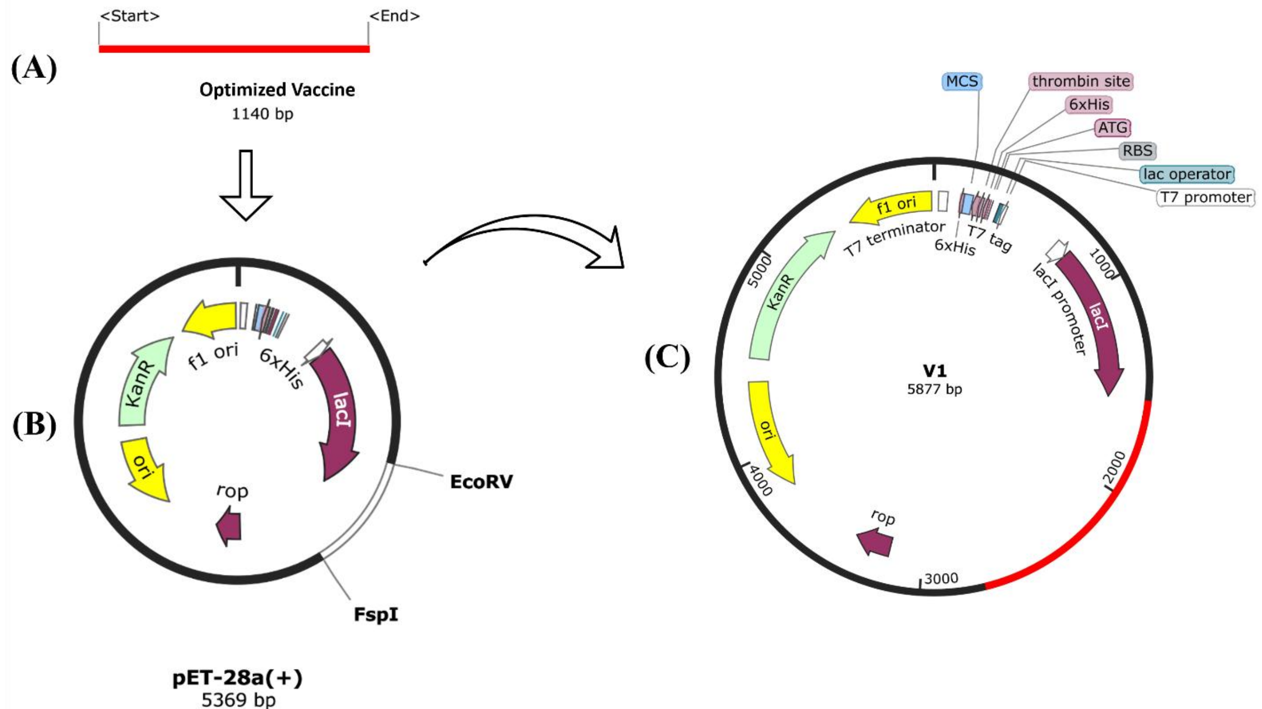

Fig. 10. In-silico cloning of developed vaccine. **(A)** Reverse-translated sequence of vaccine; **(B)** Pet28a(+) vector with the cut on restricition site; **(C)** Cloned vaccine

In current study, MEV construct was synthesized and developed using prioritized antigenic proteins to produce a highly rational and carefully engineered multi-epitope vaccine. The EAAAK linker is used because of its helical rigidity, and was placed at the appropriate location to maintain the spatial separation between functional domains, minimising unfavourable interactions thus increasing the structural stability of fusion construct. GPGPG linker was then used to reduce immunogenicity of the junctions of multi-epitope vaccines and to enhance presentation of the helper T-cell epitopes. Opting for a better proteasomal processing and MHC I, further refinement of the construct was obtained by the incorporation of AAY linkers. The addition of KK linkers, which have lysine residues with a positive charge, also enhanced the presentation of antigens, enhancing their solubility and interactions with immune receptors. The L7/L12 ribosomal protein was attached as an adjuvant to N-terminus of construct to further potentiate the immune response. Ribosomal adjuvant a powerful immunomodulator, has been shown to combine to induce innate immune responses and TLR4 through activation of pro-inflammatory pathways. This enhances antigen presentation, induces adaptive immune responses, and prime for an immune response (25). In the present study, only the vaccine construct having ribosomal protein adjuvant was docked with TLR4 to check the immunogenicity. So, this receptor was chosen for the vaccine and receptor docking simulations where vaccine was found to have the strongest interaction with the lowest binding energy.

Molecular docking analysis indicated that designed vaccine construct formed a highly stable and energetically favourable interaction with the TLR4, indicating the strong immunogenic potential of designed vaccine. This interaction was confirmed by normal mode analysis (NMA) which indicated low eigenvalues, corresponding to low energy required for deformation of the structure and, therefore, high complex stability. Immune simulation studies confirmed immunological potential of vaccine construct; in addition, comprehensive overview of expected immune response was obtained. Simulations showed long-lasting and strong immune activation, including an increase in the levels of the antibodies, immunoglobulins (IgM and IgG), an increase in the number of helper cytotoxic T cells, and an increase in production of IFN-γ and IL-2. However, repeated exposures to the antigen resulted in the formation of "immunological memory," which is an essential factor for long-term protection (26, 27). These findings indicate that designed vaccine has potential to stimulate humoral and cellular immunity and thus provide broad protection against infection by

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U. urealyticum, a critical part of the process from computational design to experimental validation and large-scale production.

CONCLUSION

To synthesize a multi-epitope subunit vaccine towards MBA protein of *Ureaplasma urealyticum*, this study employed reverse vaccinology methods and immunoinformatics. A small number of selected epitopes demonstrated strong antigenicity, safety, and coverage of the entire population. The resulting vaccine construct showed innate and adaptive immunological responses as it exhibited good physicochemical properties, stability, and good binding affinity with immune receptors. It showed immune simulation with a robust and sustained immune response and in-silico cloning and codon optimization confirmed that it could be expressed. In general, the findings indicate a promising vaccine candidate; however, in-vivo and in-vitro tests are needed to confirm its efficacy and safety.

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Conflict of interest:

Authors declared no conflict of interest.

Author s' contributions:

FA Conducted the experiments, write up and data collection; KUM Conceptualized, write up and supervised the study; MS Analysis of results, provided the experimental support and supervision.

Declaration of generative AI-Assisted Tools:

Authors declared that no AI-assisted tools were used.

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