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Immunoinformatics Approach to Designing a Subunit Vaccine Construct of *Pseudomonas aeruginosa* Outer Membrane Epitopes

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ABSTRACT

Introduction: Antibiotic-resistant *Pseudomonas aeruginosa* has been designated by the World Health Organization (WHO) as a critical priority pathogen, highlighting the critical need for developing new strategies, particularly prophylactic measures. This research focuses on incorporating highly antigenic elements from essential, surface-exposed outer membrane proteins of *P. aeruginosa* to design a polypeptide-based subunit vaccine capable of inducing a strong immune response, using immunoinformatics approaches. **Methods**: Ten essential outer membrane proteins of *P. aeruginosa* were analyzed using three online servers (ABCpred, BCPREDS, and LBtope) to predict B-cell epitopes and the IEDB server to predict CD8+ and CD4+ T-cell epitopes. The predicted epitopes were then assessed for physicochemical properties, allergenicity, and toxicity using relevant web servers. A vaccine construct incorporating the selected epitopes and an adjuvant was designed, and its 3D structure was modeled to study its interaction with Toll-like receptor-4 (TLR-4). **Results:** The final vaccine construct consisted of a 478-amino acid polypeptide incorporating 5 CD8+ T-cell, 5 CD4+ T-cell, and 15 B-cell epitopes. *In silico* analysis predicted the vaccine construct to be immunogenic, non-toxic, non-allergenic, and possess favorable physicochemical properties. Molecular docking simulations predicted strong binding affinity between the vaccine construct and TLR-4, suggesting its potential to elicit a robust immune response. **Conclusion**: These *in silico* analyses suggest that the designed subunit vaccine is potentially safe and effective against *P. aeruginosa*. However, experimental validation is necessary to confirm these predictions.

INTRODUCTION

Pseudomonas aeruginosa is an aerobic Gram-negative opportunistic pathogen. It causes high morbidity and mortality in individuals with cystic fibrosis, cancer, compromised immune systems, and burn injuries [1]. The ability to develop both intrinsic and extrinsic antibiotic resistance mechanisms, adapt to several environmental conditions, and produce a wide variety of virulence factors makes *P. aeruginosa* eradication difficult in infected patients [2]. *P. aeruginosa* is one of the six multidrug-resistant pathogens abbreviated as ESKAPE, standing for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter cloacae*. The World Health Organization (WHO) has highlighted that this group urgently needs new antibiotics in clinics [3]. The rate of *P. aeruginosa* infection in hospitalized patients, particularly those with lower respiratory tract and bloodstream infections, has increased in recent years [4]. Furthermore, the rising prevalence of multidrug-resistant *P. aeruginosa* strains may increase global mortality rates [5]. The WHO has designated antibiotic-resistant *P. aeruginosa* as a critical priority pathogen [6]. It is important to note that the inappropriate and prolonged use of antibiotics substantially contributes to the emergence of resistant strains [۷].

A crucial approach to combatting multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *P. aeruginosa* is developing a prophylactic vaccine to prevent the onset of infection. In recent years, the global spread of these high-risk strains, posing a threat to public health, has highlighted the potential of vaccines as a promising alternative to help prevent *P. aeruginosa* infections in susceptible individuals [8]. Therefore, the availability of a vaccine for this pathogen could lead to the prevention of infection and a subsequent reduction in antibiotic overuse, a major driver of antibiotic resistance

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[9]. Despite the introduction of several vaccine formulations in the past, no approved Pseudomonas vaccine has reached the market [10], highlighting the urgent need for an effective vaccine to provide effective protection against infection.

Developing a vaccine against *P. aeruginosa* has become a major challenge due to its diverse virulence factors, large genome, and the complexities of the host's immune response. Despite numerous clinical studies and investigation of various antigens over the last 50 years, advances have been made in antigen discovery, the use of adjuvants, and new delivery systems for vaccine platforms. Several vaccine candidates have progressed to clinical trials, but none have yet received approval for commercial use [10].

In contrast to early vaccine development efforts against *P. aeruginosa*, which primarily focused on killed or attenuated whole-cell vaccines, current research emphasizes subunit vaccines composed of specific immunogenic epitopes. Outer membrane proteins, particularly adhesins, are key targets for vaccine development because they are exposed on the bacterial surface and interact directly with the host immune system in Gram-negative bacteria. While some outer membrane proteins, such as OmpA and OprF, have shown promise as vaccine candidates, focusing on a limited number of antigens may restrict the breadth and efficacy of the induced immune response. Given the lengthy and costly process of traditional vaccine development, which can take at least ten years from basic research to commercial production [11], immunoinformatics approaches offer a promising alternative for accelerating vaccine design and development [12, 13].

In the present study, we used ten essential outer membrane proteins of *P. aeruginosa* (Table 1) as the source proteins to identify antigenic elements capable of inducing both B-cell and T-cell immunity. By rationally combining these peptides and incorporating an adjuvant to enhance the host's specific antigen immune response, we created a vaccine polypeptide with minimal toxicity and allergenicity. This rationally designed vaccine construct holds significant promise for eliciting a potent and broad immune response against *P. aeruginosa*. Further experimental studies are warranted to validate the immunogenicity and protective efficacy of this vaccine construct.

METHODS

To design a subunit vaccine against *Pseudomonas aeruginosa*, epitopes that stimulate CD4+ and CD8+ T cells, as well as B cells, were selected. The allergenicity, toxicity, and physicochemical properties of these epitopes were evaluated using various web servers. Subsequently, the binding affinity of the epitopes to the TLR4 receptor was assessed using molecular docking tools, specifically HDOCK and ClusPro 2.0. The flowchart involved in designing the vaccine construct is presented in Figure 1.

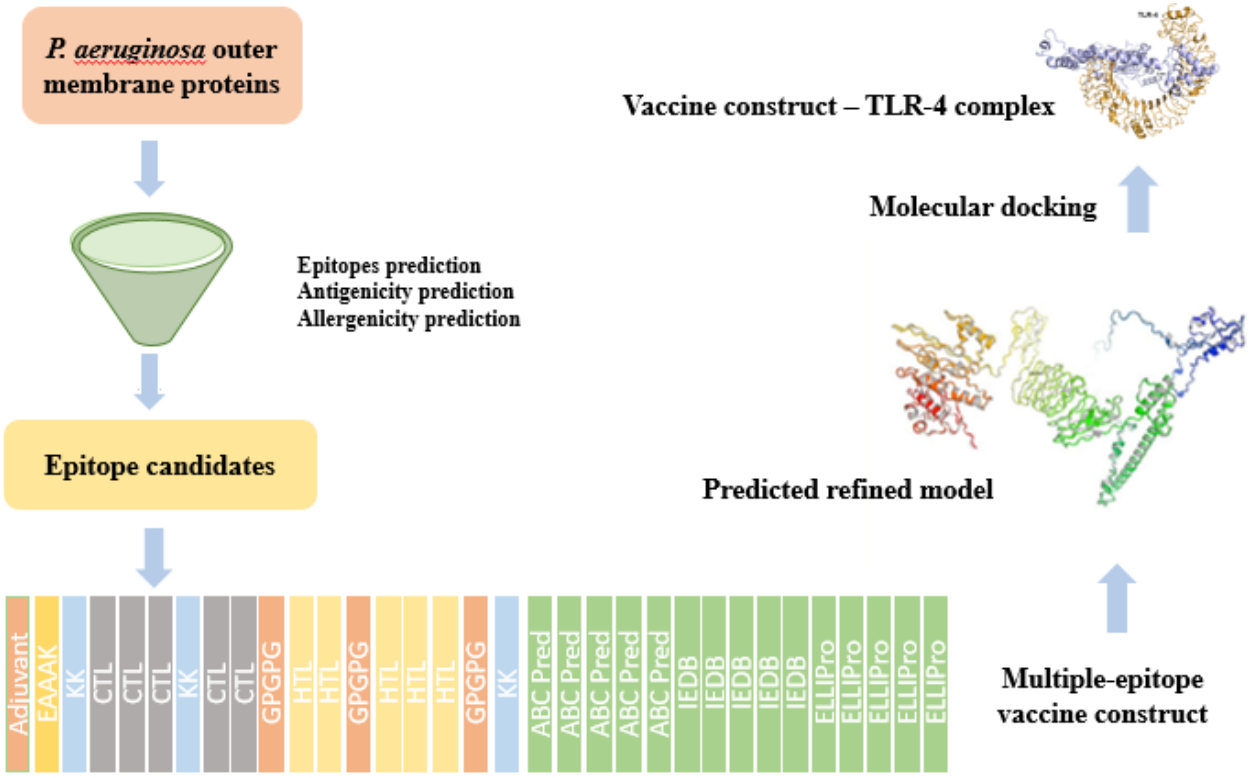


Fig. 1. Schematic workflow for designing the multi-epitope vaccine against *P. aeruginosa*.

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Protein sequences. The FASTA sequences of ten essential outer membrane proteins from *P. aeruginosa*, selected based on their high conservation, predicted immunogenicity, and role in pathogenesis, were retrieved from the NCBI and Pseudomonas Genome Database (Table 1).

Table 1. Computed characteristics of the studied outer membrane proteins of *P. aeruginosa*

Protein name	MW (kDa)	pI	Instability index	Half-life in mammals (hr)	Half-life in *E. coli* (hr)	Aliphatic index	Grand average of hydropathicity (GRAVY)
Iron receptor	82.34	5.72	22.18	30	> 10	66.10	-0.594
PilY1	126.58	6.00	29.21	30	> 10	67.05	-0.500
FpvB	87.43	5.60	27.31	30	> 10	71.96	-0.455
FiuA	88.21	5.46	34.78	30	> 10	72.51	-0.480
OprF	37.64	4.98	26.16	30	> 10	69.94	-0.443
Ferric receptor	80.97	5.65	36.81	30	> 10	74.18	-0.557
OpdP	53.03	5.61	24.39	30	> 10	70	-0.484
Hypothetical protein PA1288	45.56	5.73	18.92	30	> 10	78.231	-0.263
OprO	47.79	5.17	17.64	30	> 10	64.703	-0.499
OprE	49.67	8.67	29.95	30	> 10	72.96	-0.436

Prediction of B cell epitopes. B cell epitopes, which are recognized by B lymphocytes to initiate humoral immunity, are crucial for vaccine design due to their role in inducing antibody production and antigen neutralization [١٤]. ABCpreds (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html) and the Immune Epitope Database (IEDB) server were used to predict 16-mer linear B cell epitopes. ABCpreds utilized a threshold of 0.51, while IEDB employed Bepipred Linear Epitope Prediction 2.0. Continuous B cell epitopes were predicted using the ElliPro tool within the IEDB. To maximize the coverage of predicted epitopes, three different servers (ABCpreds, IEDB, and ElliPro) were employed.

Prediction of cytotoxic T lymphocyte (CTL) epitopes. Predicting peptides capable of inducing cytotoxic T lymphocyte (CTL) responses is crucial for epitope-based vaccine design, as CTLs recognize and eliminate infected cells presenting specific antigens [15]. CD8+ T-cell epitopes within the 10 outer membrane proteins were predicted using the MHC-I Binding tool of the IEDB (http://tools.iedb.org/mhci/). The prediction method employed at this stage was ANN 4.0. Human was selected as the source species. The analysis utilized the IEDB recommended set for human MHC-I alleles. The maximum IC50 value was set to 50 nM, and a percentile rank <1 was considered the threshold for high-affinity binding, as lower percentile ranks indicate stronger binding.

Prediction of helper T lymphocyte (HTL) epitopes. MHC-II binding of 15-mer epitopes within the 10 outer membrane proteins was predicted using the IEDB server (http://tools.iedb.org/mhcii/). The prediction was performed using the NetMHCII 2.2 method, with human selected as the source species. The analysis utilized the full HLA reference set provided by IEDB. Epitopes were classified based on their predicted IC50 values: <50 nM (high affinity), 50-500 nM (intermediate affinity), and >500 nM (low affinity). Only 15-mer epitopes with high affinity (IC50 < 50 nM) were considered for inclusion in the vaccine polypeptide.

Evaluation of identified epitopes for antigenicity, allergenicity and toxicity. The antigenic potential of each predicted T and B cell epitope was evaluated using VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen_citation.html), a server for alignment-independent prediction of protective antigens based on the physicochemical properties of proteins [16]. Bacteria was selected as the target organism, and other parameters were set to default values [17]. The toxicity and allergenicity of each epitope were predicted using ToxinPred (http://crdd.osdd.net/raghava/toxinpred/multi_submit.php) [18, 19], and and AllergenFP v1.0 [20].

Designing of multi-epitope vaccine polypeptide construct. The final vaccine structure was designed by selecting a combination of predicted CTL, HTL, and LBL epitopes. Epitopes were selected that had high antigenic potential and were not identified as allergenic or toxic. The final construct comprised 20 epitopes, including 5 CTL, 5 HTL, and 15 LBL epitopes, joined by KK and GPGPG linkers.

Evaluation of vaccine construct for antigenicity, allergenicity and physicochemical properties. The allergenicity of the vaccine construct was predicted using the AllerTOP v.2.0 and AllergenFP v.1.0 servers. Antigenicity was predicted using the VaxiJen v.2.0 tool, employing a threshold value of 0.4. Various physicochemical properties of the vaccine construct, including (amino acid composition, instability index, total hydropathic mean (GRAVY), aliphatic index, theoretical pI and molecular weight …) were predicted using the ProtParam server.

Modeling, refinement and validation of the vaccine construct. The secondary structure of the vaccine construct, which describes the local conformation of the polypeptide backbone, was predicted using the SOPMA server [21]. This server predicts various secondary

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structure elements, including α-helices, β-strands, turns, and random coils. The 3D structure of the construct was modeled and refined using the GalaxyWEB server. The refined 3D model was then validated using the RAMPAGE and ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) servers. The ProSA server was used to assess the overall quality of the 3D model by calculating a z-score, which indicates the deviation of the model's energy from that of experimentally determined structures. Lower z-scores indicate better model quality.

Molecular docking of the vaccine construct with TLR-4. To elicit an effective immune response, the vaccine construct must bind efficiently to immune cell receptors. To assess this interaction, the binding pattern of the construct with TLR4 was examined through molecular docking analysis using the HDOCK and ClusPro 2.0 servers. Both HDOCK and ClusPro 2.0 require input files in PDB format for the receptor (TLR4) and ligand (vaccine construct). TLR4 plays a crucial role in recognizing antigens and activating the immune system, including the induction of cytokines [22, 23]. The docking analysis aimed to identify the most favorable binding configuration of the vaccine construct with TLR4 based on the lowest binding energy and highest binding efficiency. Both HDOCK and ClusPro 2.0 employ scoring functions based on a weighted sum of various energy terms, including electrostatic energy, van der Waals energy, desolvation energy, and restraints violation energy [24]. The 3D structure of TLR4 (PDB ID: 3FXI) was retrieved from the Protein Data Bank.

RESULTS

Retrieval and characterization of outer membrane protein sequences. Table 1 presents the *in silico* characterization of the ten outer membrane proteins from *P. aeruginosa* selected for vaccine design. The amino acid sequences of these proteins were retrieved from the NCBI and Pseudomonas Genome Database (Table 1).

Prediction of B cell epitopes in *P. aeruginosa* outer membrane proteins. B cell epitopes were predicted using three different servers: ABCpred, IEDB, and ElliPro. A total of 30 linear and conformational B cell epitopes were selected based on their high antigenicity, non-allergenic, and non-toxic properties. Table 2 lists the final selected linear and conformational B cell epitopes from the outer membrane proteins of *P. aeruginosa*. The complete list of predicted epitopes and their properties is provided in Online Resource 1, Table S1.

Table 2. Selected linear and conformational B cell epitopes

Linear B cell epitope prediction server	Outer membrane protein[a]	Epitope sequence	Starting position	Antigenicity score (VaxiJen v2.0)	Potential issues[b]
ABCpred	FpvB	DGHGGWGSLFNKDGTP	470	0.4958	NA, NT
ABCpred	OprE	YGSGVTKGEVDNRAFS	293	1.2739	NA, NT
ABCpred	FpvB	YSYTDDHDSYDAYANG	432	0.7788	NA, NT
ABCpred	FpvB	QGSVGTWDRYRSEADV	268	0.4029	NA, NT
ABCpred	PilY1	KVTIEYGRGYDKESTI	138	0.7818	NA, NT
IEDB B-cell	OprF	TPGVGLR	109	2.8035	NA, NT
IEDB B-cell	OpdP	PKAGGGSQRIH	91	2.5752	NA, NT
IEDB B-cell	IroN	YAITSGGGAGNT	408	2.3002	NA, NT
IEDB B-cell	OprF	NINSDSQGRQQ	133	2.2664	NA, NT
IEDB B-cell	PilY1	CNQMGSGSSSGN	231	2.2614	NA, NT
ElliPro	IroN	MSRQSTDTAVSSQRLLASAIGVAITAIAAPQAAHADEAGQKKTDKDRVLSL DAATIVG	1	0.7225	NA, NT
ElliPro	OprO	MIRKHSLGFVASALALAVSAQAFAGTVTTDGADIVIKT	1	0.4606	NA, NT
ElliPro	FepA	GLSPVDHASGGKGDYANAAIYQ	536	0.9447	NA, NT
ElliPro	OprF	DVRGTYETGNKKV	76	1.7475	NA, NT
ElliPro	PA1288	ATNGLLGSNGD	183	0.7410	NA, NT

[a] FpvB: Second ferric pyoverdine receptor FpvB; OprE: Outer membrane protein OprE; PilY1: Type 4 fimbrial biogenesis protein PilY1; OprF: Outer membrane protein F; OpdP: Glycine-glutamate dipeptide porin OpdP; IroN: Iron transport outer membrane receptor; OprO: Pyrophosphate-specific outer membrane porin OprO; FepA: Ferric enterobactin receptor; PA1288: Hypothetical protein PA1288. [b] NA: Non-allergenic; NT: Non-toxic

Prediction of T cell epitopes in *P. aeruginosa* outer membrane proteins. A total of 30 cytotoxic T lymphocyte (CTL) epitopes and 30 helper T lymphocyte (HTL) epitopes were selected based on their high antigenicity scores, non-allergenic, and non-toxic properties. Table 3 lists the selected CTL epitopes from the outer membrane proteins of *P. aeruginosa*. Further details are provided in Online Resource 1, Table S2. Table 4 presents the selected 15-mer HTL epitopes and their MHC-II binding results (Table 4). Further details are available in Online Resource 1, Table S3.

Selection of epitopes for vaccine design. T-cell and B-cell epitopes were selected for inclusion in the multi-epitope vaccine construct based on the following criteria: (1) high antigenicity predicted by VaxiJen v2.0, (2) non-allergenic nature predicted by AllerTOP v2.0 and AllergenFP v1.0, and (3) non-toxic nature predicted by ToxinPred.

Design and characterization of the multi-epitope vaccine construct. The multi-epitope vaccine construct was designed by fusing 5 CTL, 5 HTL, and 15 LBL epitopes respectively. KK and GPGPG linkers were used

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to join the epitopes as shown in Figure. To enhance immunogenicity, the β-defensin adjuvant (GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK) was added to the N-terminus of the construct using an EAAAK linker. The final construct comprised a total of 478 amino acids. Assessment of the quality of the final structure revealed an acceptable model in comparison with benchmarked experimental data (Figure 2) and a reliable structure in terms of secondary and tertiary conformations (Figure 2). The quality and potential errors in the final vaccine 3D model were verified by ProSA-web. The Z-score, which indicates the overall quality of the model, was: -2.71. However, a model with a lower Z-score is considered a higher-quality model. In addition, a plot was drawn to check the quality of local model, where negative values indicate that there is no error in the model structure (Figure 3 A-C).

Table 3. Selected cytotoxic T lymphocyte (CTL) epitopes

Outer membrane Protein[a]	Epitope sequence	MHC-I allele	Antigenicity score (VaxiJen v2.0)	Potential issues[b]
OpdP	GLSTGVWYV	HLA-A*02:01	0.5218	NA, NT
FiuA	EVKAEVGNY	HLA-A*26:01	1.0402	NA, NT
IroN	NVANGYVYR	HLA-A*68:01	0.9664	NA, NT
IroN	TPTRATLSY	HLA-B*35:01	0.4929	NA, NT
OprO	YYAQLAYTL	HLA-A*24:02	0.8250	NA, NT

[a] OpdP: Glycine-glutamate dipeptide porin OpdP; FiuA: Ferrichrome receptor FiuA; IroN: Iron transport outer membrane receptor; OprO: Pyrophosphate-specific outer membrane porin OprO.
[b] NA: Non-allergenic; NT: Non-toxic.

Table 4. Selected helper T lymphocyte (HTL) epitopes

Outer Membrane Protein[a]	Epitope Sequence	MHC-II Binding Core	IC50 (nM)	Antigenicity Score (VaxiJen v2.0)	MHC-II Allele	Potential Issues[b]
OprO	GVNWYVNDAVKISAA	WYVNDAVKI	2.4	0.6078	HLA-DRB3*02:02	PNA, NT
PilY1	STKQSGWYLDFMVNG	KQSGWYLDF	6.7	0.8237	HLA-DQA1*01:01/DQB1*05:01	PNA, NT
PilY1	GAHQFYVDGSPVVAD	FYVDGSPVV	2.8	0.5030	HLA-DRB3*01:01	PNA, NT
OprO	VASALALAVSAQAFA	LALAVSAQA	6.6	0.4801	HLA-DRB1*09:01	PNA, NT
PilY1	KESTIKADAAYYYDF	IKADAAYYY	3.1	0.8039	HLA-DRB3*01:01	PNA, NT

[a] OprO: Pyrophosphate-specific outer membrane porin OprO; PilY1: Type 4 fimbrial biogenesis protein PilY1.
[b] PNA: Probable non-allergenic; NT: Non-toxic.

Predicted properties of the vaccine construct. The final vaccine construct was evaluated for its immunogenicity, allergenicity, and toxicity. The VaxiJen server predicted an antigenicity score of 0.8975, which is above the threshold of 0.4 for bacterial proteins. The construct was predicted to be non-allergenic and non-toxic, suggesting a suitable design. The calculated molecular weight of the construct was 50.13 kDa, which is within the desirable range for vaccine antigens, and the pI value of 9.26 indicated its basic nature. The predicted physicochemical properties of the construct are summarized in Table 5. The instability index (24.31) suggests high stability, while the aliphatic index (65.19) indicates high thermal stability. The construct was predicted to be slightly hydrophilic (average hydrophilicity: -0.472). The estimated half-life was 30 h in mammalian reticulocytes *in vitro*, >20 h in yeast, and >10 h in *E. coli in vivo*.

Table 5. Physicochemical properties and predicted characteristics of the final vaccine construct.

Features	Assessment
Number of amino acid residues	478
Molecular weight	50.137 kDa
Theoretical pI	9.26
Total number of negatively charged residues (Asp + Glu)	40
Total number of positively charged residues (Arg + Lys)	55
Extinction coefficient (ε_{280}, $M^{-1}cm^{-1}$)	69595
Instability index	24.31
Aliphatic index	65.19
Antigenicity (PREDICTED TOOL)	0.8975
Allergenicity	AllerFP v.1.0: Non-allergen AllerTOP v.2.0: Non-allergen
Solubility probability (SOLpro)	0.852
Grand average of hydropathicity (GRAVY)	-0.472

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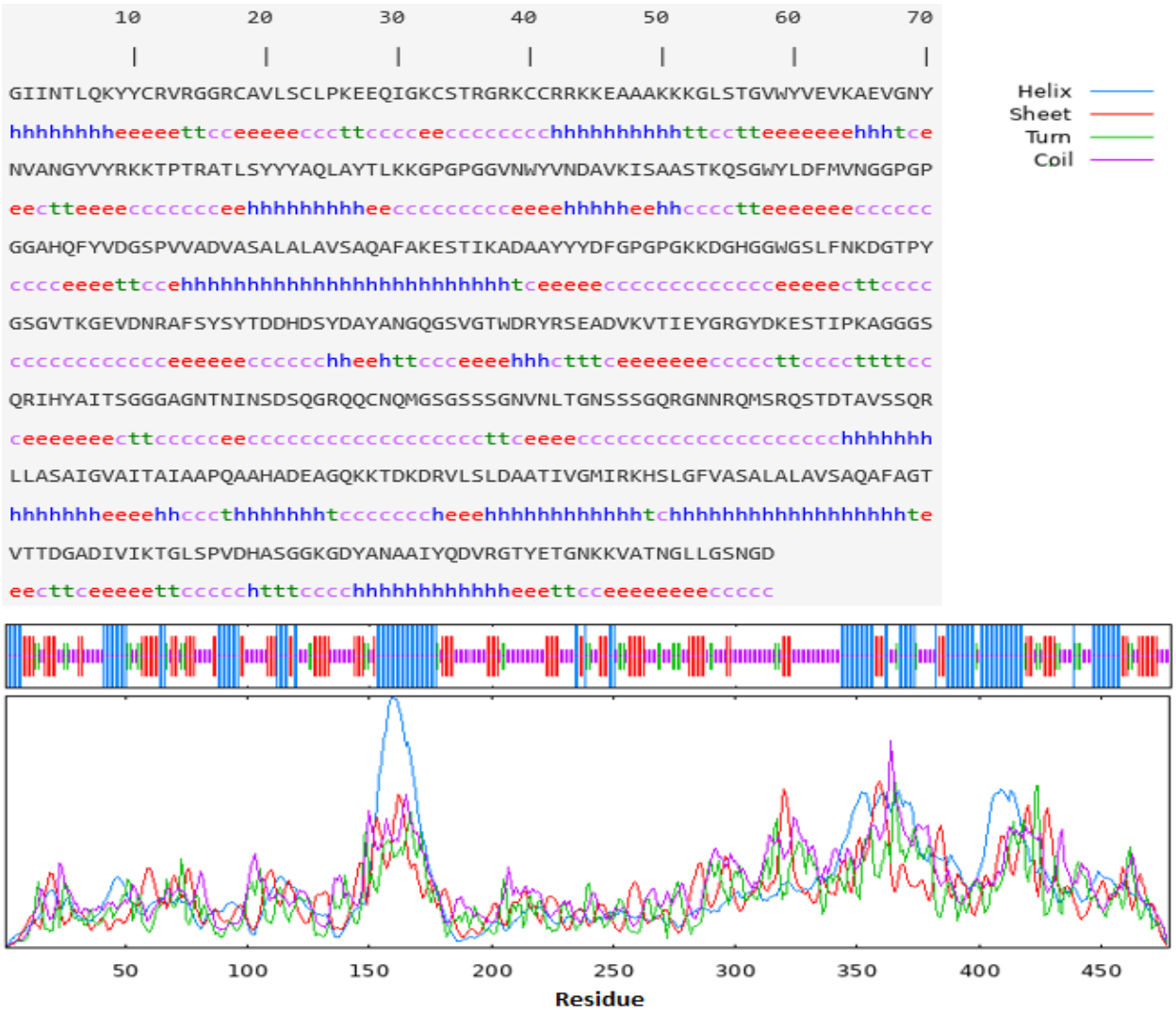


Fig. 2. The final sequence of the construct and its secondary structural profile obtained by SOPMA.

Prediction and validation of the 3D structure of the vaccine construct. The three-dimensional structure of the vaccine construct was predicted using GalaxyWEB and refined using energy minimization and loop modeling. The quality of the predicted 3D structure was validated using ProSA and RAMPAGE. The ProSA Z-score of -2.71 indicates that the model's energy is within the range of experimentally determined structures, suggesting a good overall quality. The Ramachandran plot analysis showed the high quality of the designed construct (Figure 3).

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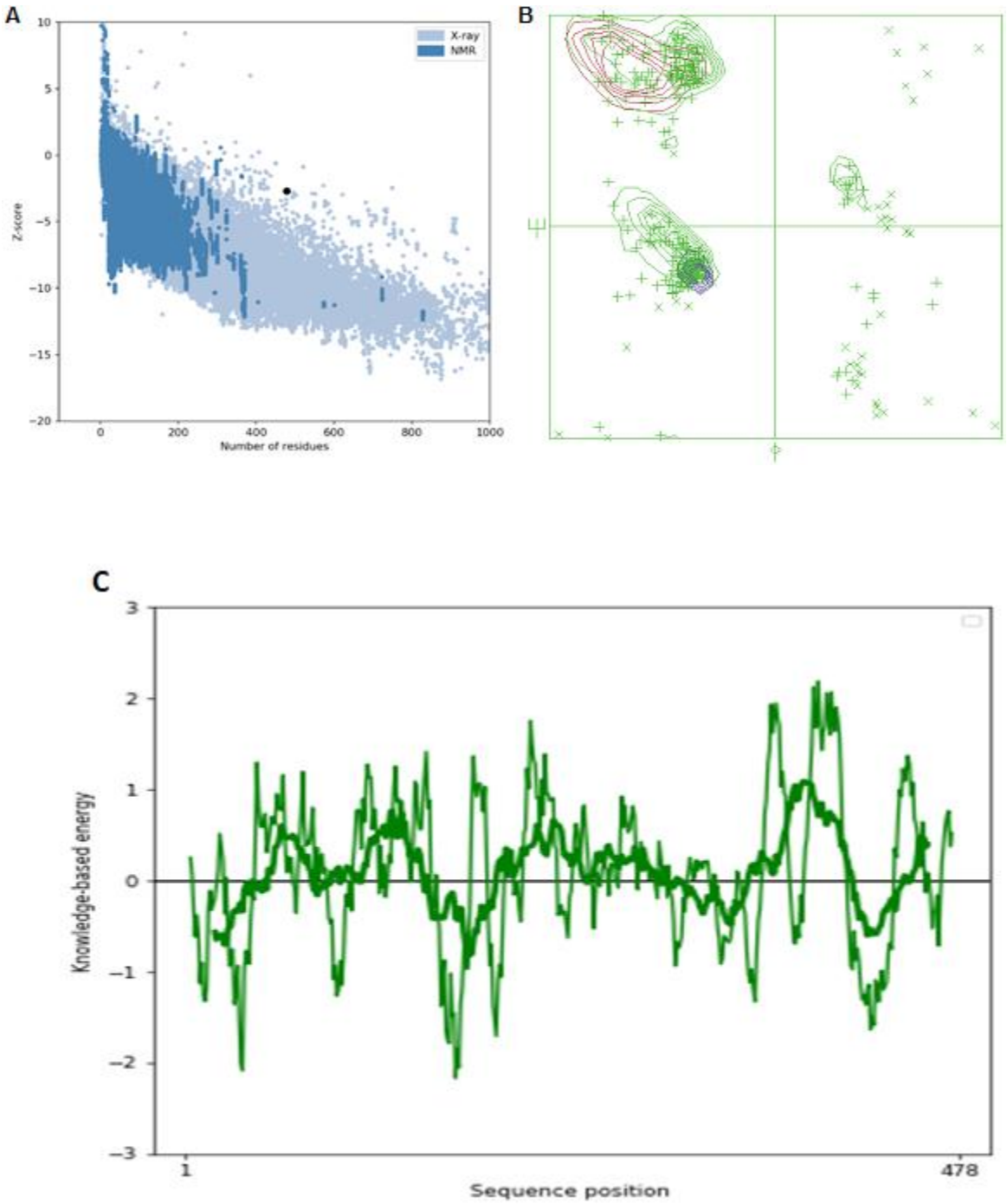


Fig 3. Validation of the vaccine 3D structure by ProSA-web (A) and RAMPAGE (B). Based on ProSA-web, the Z-score of the refined model was -2.71. The quality of local model was checked by negative values indicating that there is no error in the model structure (C)

Molecular docking analysis of the vaccine construct with TLR4. The best docking configuration of the vaccine construct with TLR4 was selected based on the lowest binding free energy obtained from ClusPro and HDOCK. Comparison of the docking results from ClusPro and HDOCK revealed similar binding interfaces between the vaccine construct and TLR4 (Figure 4). Analysis of the interacting residues at the binding interface (Table 6) revealed a prominent role of polar residues from the vaccine construct and non-polar, positively-charged residues from TLR4 in mediating the interaction. The binding energy scores obtained from ClusPro v2.0 and HDOCK were -889.5 kcal/mol and -334.58 kcal/mol, respectively. These values suggest a strong interaction between the vaccine construct and

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TLR4, as they are comparable to or lower than typical binding energies observed for protein-protein complexes.

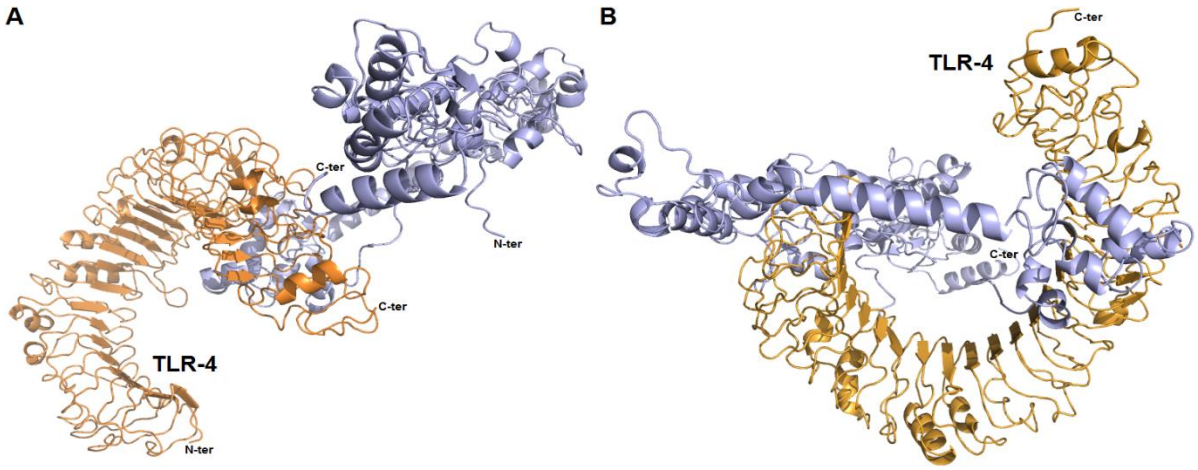


Fig. 4 Interactions between TLR-4 (orange) and the designed vaccine construct (blue), as predicted by ClusPro (A) and HDOCK (B).

Table 6. Interacting residues at the TLR4-vaccine construct interface

Vaccine construct residue	TLR4 residue	Minimum distance (Å)
Q242	F75	1.882
N109	G480	2.003
A239	F75	2.129
N113	Q507	2.137
Y238	D50	2.267
Y238	F75	2.37
K117	Q507	2.409
Y76	R382	2.64
Q242	P78	2.655
Y78	H431	2.662

DISCUSSION

The rise of antibiotic resistance necessitates the development of novel strategies to combat bacterial pathogens, making it a critical health priority. While traditional live or attenuated vaccines have significantly reduced morbidity and mortality from various infectious diseases, their development and production can be challenging. These challenges include the potential for autoimmune or allergic reactions, as well as the high costs and long timelines associated with their development. Peptide-based vaccines offer a promising alternative that could potentially address some of these limitations [25]. Bioinformatics approaches, utilizing various databases and servers, have recently emerged as a cost-effective, rapid, and reliable method for predicting antigenic regions for subunit vaccine development [26]. The increasing availability of genomic and proteomic data, coupled with advances in immunoinformatics techniques, has facilitated the development of vaccines against resistant and life-threatening pathogens [27, 28]. Given the urgent need for an effective vaccine against *P. aeruginosa*, this study aimed to leverage immunoinformatics approaches to identify potential vaccine candidates within the pathogen's proteome. Specifically, we focused on outer membrane proteins due to their accessibility to the immune system and their role in pathogenesis.

This study employed an immunoinformatics approach to design a potential vaccine against *P. aeruginosa* by targeting 10 outer membrane proteins. B and T cell epitopes were predicted using a combination of three servers, including ABCpred, ElliPro and IEDB. The most promising epitopes were selected based on their predicted antigenicity, allergenicity, toxicity, and binding affinity to MHC molecules.

Outer membrane proteins are crucial for bacterial survival and pathogenesis. They contribute to various functions, including: (1) adaptation to high osmolarity environments, (2) evasion of host immune responses, such as by interfering with macrophage phagocytosis, and (3) modulation of bacterial sensitivity to antimicrobial peptides [26]. Furthermore, as outer membrane proteins are at the forefront of host-pathogen interactions, they represent promising targets for vaccine development [27, 29]. Previous studies have explored outer membrane proteins and iron uptake proteins, either individually or in combination, as potential vaccine targets against various bacterial pathogens, including *P. aeruginosa* [28, 29]. For

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example, Irum *et al*. (2021) designed a multi-epitope vaccine targeting five outer membrane proteins of *P. aeruginosa* to elicit both humoral and cellular immune responses against the pathogen [30]. Furthermore, studies have demonstrated the ability of epitopes derived from outer membrane proteins, such as (such as OprF/OprI hybrid protein as a vaccine candidate against *P. aeruginosa*), to induce antibody production and protective immunity in both human and animal models [31]. Mansouri *et al*. (2003) conducted a phase I/II human clinical trial of a vaccine based on the OprF-OprI epitopes [31]. In contrast, this study utilized bioinformatics tools to design a novel multi-epitope vaccine against *P. aeruginosa* by incorporating predicted immunogenic epitopes from ten outer membrane proteins selected for their potential role in pathogenesis.

Ideally, vaccine epitopes should exhibit broad binding affinity to multiple MHC class I and class II alleles, ensuring effective presentation to a wider range of immune cells and potentially enhancing population coverage [32]. Based on a rigorous screening process that considered predicted antigenicity, allergenicity, toxicity, and MHC binding affinity, a total of 25 epitopes were selected for vaccine design: 15 B cell epitopes, 5 MHC class I binding epitopes, and 5 MHC class II binding epitopes. These epitopes were selected based on their predicted immunogenicity, non-toxicity, and non-allergenic nature, ensuring their safety and efficacy in the host. The final vaccine construct was assembled by combining the selected epitopes with a β-defensin adjuvant and specific linkers. An epitope-based vaccine design offers several advantages over whole-protein vaccines, including targeted delivery of antigenic regions and a reduced risk of allergic reactions [33]. However, individual epitopes may have limited immunogenicity. To overcome this limitation, we incorporated multiple epitopes from all 10 target proteins into a single construct, creating a multi-epitope vaccine with potentially enhanced immunogenicity [34]. The designed vaccine construct was evaluated for its antigenicity, allergenicity, toxicity, and physicochemical properties. *In silico* characterization revealed a low molecular weight, facilitating purification, and a basic isoelectric point (pI) due to a higher proportion of positively charged amino acids. The high aliphatic index suggests high thermostability, while the negative GRAVY value indicates a hydrophilic nature, promoting strong interactions with water molecules. The instability index (less than 40.00) and predicted solubility suggest a stable protein construct. These predicted properties suggest that the construct is likely to be stable and soluble *in vivo*. The Ramachandran plot analysis further supports the structural validity of the model, with a high percentage of residues falling within favorable regions. Molecular docking analysis using ClusPro 2.0 and HDOCK predicted a strong interaction between the vaccine construct and TLR4, suggesting its potential to effectively activate the immune system. These findings strongly suggest that the designed subunit vaccine construct is a promising candidate for further development and experimental validation in preclinical models.

Vaccine development is a time-consuming and costly process, from initial research to approval and deployment. Bioinformatics approaches can significantly accelerate the identification of protective antigens for vaccine development. However, rigorous preclinical evaluation, including *in vitro* and *in vivo* studies, is essential to assess the safety and immunogenicity of these vaccine candidates. Furthermore, bioinformatics can contribute to strategies addressing the global challenge of antibiotic resistance. Notably, these approaches hold promise for developing vaccines specifically targeting antibiotic-resistant pathogens. While the application of bioinformatics to vaccine development for bacterial pathogens is still emerging, it has demonstrated promising results in several cases [11, 35]. While this study focused on 10 outer membrane proteins of *P. aeruginosa*, selected using reverse vaccinology methods to introduce suitable vaccine candidate proteins against *P. aeruginosa* outer membrane proteins (OMPs) [36], rather than encompassing the entire proteome, this immunoinformatics approach offers a potentially cost-effective, time-efficient strategy for vaccine design, with the potential for improved accuracy in identifying promising vaccine candidates compared to traditional experimental methods. Further research incorporating a larger set of outer membrane proteins is warranted to ensure comprehensive coverage of potential antigens.

A primary limitation of this study is the lack of experimental validation of the predicted immunogenicity and protective efficacy of the designed vaccine construct. This was beyond the scope of the current *in silico* study. Future studies should prioritize *in vitro* and *in vivo* experiments to validate the *in silico* predictions. These could include assessing the immunogenicity of the vaccine construct in animal models using techniques such as ELISA, evaluating its ability to elicit protective immune responses against *P. aeruginosa* challenge, and determining its safety profile through histological analysis and other relevant assays. These experiments will be crucial for advancing the development of this vaccine candidate towards clinical trials

In this study, a multi-epitope subunit vaccine was designed *in silico*, targeting predicted immunodominant epitopes from *P. aeruginosa* outer membrane proteins. The potential efficacy of this vaccine construct was then evaluated using various immunoinformatics tools, focusing on predicted immunogenicity and MHC binding affinity. The *in silico* analysis predicted that this subunit vaccine construct could potentially elicit robust humoral and cellular immune responses. However, it is crucial to acknowledge that these are preliminary *in silico* predictions, and experimental validation is essential to

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confirm the actual immunogenicity and protective efficacy of the vaccine construct.

Further research is warranted to validate the efficacy and safety of this vaccine construct. This includes preclinical studies in animal models to assess immunogenicity and protection against *P. aeruginosa* infection. Exploring alternative vaccine platforms, such as mRNA vaccines, and the use of delivery systems, such as lipid nanoparticles, could also be valuable avenues for future investigation. The findings of this study provide a promising starting point for the development of a novel vaccine against *P. aeruginosa*, but further research is needed to translate these *in silico* predictions into a viable vaccine candidate.

CONFLICT OF INTEREST

The author declares that she has no conflicts of interest associated with this manuscript.

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