Developing a Putative Vaccine against the Methicillin-Resistant *Staphylococcus aureus* using Immunoinformatic

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Abstract

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) is recognized as one of the most critical antibiotic-resistant pathogens worldwide and represents a major challenge to global public health systems. Its ability to resist multiple classes of antibiotics has led to increased morbidity, mortality, and healthcare costs. The limited effectiveness of current therapeutic options emphasizes the urgent need for alternative preventive strategies, among which vaccine development is considered a promising and sustainable approach.

Objective:

The present study aimed to design, construct, and evaluate a rational multi-epitope vaccine candidate against MRSA by integrating immunoinformatics-based prediction tools with experimental in vivo validation.

Materials and Methods: Seven MRSA virulence-associated proteins were initially screened for antigenicity, immunogenicity, and allergenicity using established bioinformatics databases and servers. Highly immunogenic B-cell and T-cell epitopes were selected and assembled using suitable peptide linkers to ensure structural stability and optimal epitope presentation. An immunostimulatory adjuvant was fused to the N-terminal region of the construct to enhance immune responses. Physicochemical properties, including molecular weight, isoelectric point, stability index, and hydropathicity, were analyzed using ProtParam. Secondary and tertiary structures were predicted and refined using Prabi, Robetta, AlphaFold, and I-TASSER servers. Antigenicity and allergenicity were evaluated using VaxiJen and AllerTOP, respectively. Molecular docking analyses with Toll-like receptor 4 (TLR4) and selected human leukocyte antigen (HLA) alleles were performed using Molegro Virtual Docker to assess binding affinity and immune receptor interactions. The optimized gene sequence was cloned into the pcDNA3.1 expression vector and administered to BALB/c mice. The expression levels of

immune-related genes, including TLR4 and interleukin-4 (IL-4), were quantified using real-time PCR.

Results: Computational analyses demonstrated strong binding interactions between the vaccine construct and immune receptors, indicating a high potential for immune activation. The vaccine candidate was predicted to be non-allergenic and antigenic. In vivo experiments revealed a significant downregulation of TLR4 and IL-4 gene expression in immunized mice compared with control groups.

Conclusion: The designed multi-epitope vaccine candidate exhibits favorable immunological and structural characteristics against MRSA. Although these findings are promising, further comprehensive in vitro and in vivo investigations are required to confirm its protective efficacy, immunogenicity, and long-term safety.

Keywords: immunoinformatic, in silico vaccine developing, multi-epitope vaccine, *Staphylococcus aureus*

1. Introduction

The rapid emergence and widespread spread of highly virulent, multidrug-resistant (MDR) *Staphylococcus aureus* strains have rendered invasive infections caused by this bacterium a leading contributor to morbidity and mortality in both hospital and community environments. The acquisition of resistance to newly developed antibiotic classes is made easier by the pathogen's pronounced genetic adaptability, mostly through horizontal gene transfer (HGT). Through this process, *Staphylococcus aureus* incorporates exogenous genetic material from neighboring microorganisms, thereby gaining multiple adaptive benefits—most notably the uptake of preformed resistance determinants that confer concurrent protection against a broad spectrum of antibiotics. The emergence of methicillin- and vancomycin-resistant

Staphylococcus strains is largely driven by horizontal gene transfer. Moreover, the widespread resistance observed against antibiotics such as vancomycin, daptomycin, fluoroquinolones, and linezolid is largely attributable to intrinsic resistance mechanisms resulting from spontaneous genetic mutations, followed by the selective persistence of bacterial populations under sustained antibiotic pressure (1).

In modern clinical practice, growing attention is being directed toward the development of immune-based approaches to control *Staphylococcus aureus* infections, thereby reducing reliance on antibiotic therapy alone. A significant global health threat is presented by the rapid rise of antibiotic-resistant *Staphylococcus aureus* strains, which highlights the urgent need for an effective vaccine that can target multidrug-resistant variants. There is currently no licensed vaccine available to prevent *Staphylococcus aureus* infections. The majority of previous research has concentrated on assessing the immunogenic abilities of iron-regulated surface determinant A (IsdA) and capsular polysaccharides as potential vaccine antigens. While these components demonstrated encouraging protective effects in murine models, comparable efficacy has not yet been confirmed in human clinical trials (2, 3). Reverse vaccinology represents an advanced and rational approach to vaccine design that leverages genomic and proteomic data to identify and incorporate multiple antigenic epitopes capable of eliciting a precise and robust immune response. Reverse vaccinology constitutes a rational and advanced strategy for vaccine development that exploits genomic and proteomic information to identify and integrate multiple antigenic epitopes capable of inducing a targeted and robust immune response (4). Although an epitope represents only a limited structural component of an antigen, it is essential for immune recognition and is capable of inducing highly specific and potent immune responses while reducing off-target effects. In recent years, substantial emphasis has been placed on the development of multi-epitope vaccines that simultaneously address multiple

pathogenic mechanisms, thereby improving both the precision and overall effectiveness of immune protection (5).

Several immunoinformatics-based studies have previously investigated the design of multi-epitope vaccines targeting *Staphylococcus aureus*. For instance, Solanki *et al.* employed subtractive proteomics in combination with reverse vaccinology to prioritize extracellular antigens and proposed a multi-epitope vaccine construct for protection against *S. aureus* (6), whereas Thomas and Doytchinova described a systematic in silico workflow for the identification of B- and T-cell epitopes derived from staphylococcal proteins (7). Comparable pipelines have been applied to other priority pathogens—including *Klebsiella pneumoniae* (8), *Pseudomonas aeruginosa* (9), and *Mycobacterium tuberculosis* (10)—underscoring the generalizability of epitope-centric vaccine design across Gram-negative and acid-fast bacteria.

Among current vaccine development strategies, multi-epitope constructs have emerged as particularly promising candidates. These vaccines are intrinsically non-pathogenic, cost-effective, and suitable for large-scale production. By facilitating antigen presentation through both major histocompatibility complex (MHC) class I and class II pathways, they elicit robust and well-balanced immune responses. Moreover, the incorporation of multiple antigenic determinants within a single construct enables multi-epitope vaccines to induce broad-spectrum and comprehensive immune protection, thereby enhancing their efficacy across diverse pathogenic settings (11). In the present study, an in silico immunoinformatics approach was employed to identify promising antigenic targets and to rationally design a multi-epitope vaccine candidate against methicillin-resistant *S. aureus* (MRSA).

2. Materials and Methods

2.1. Protein Selection

Seven virulence-associated proteins (Table 1) were chosen based on previous studies focusing on MRSA vaccine development. The seven proteins were selected based on their reported surface localization, virulence contribution, and previous evidence of immunogenicity in MRSA vaccine studies. Although other virulence factors such as enterotoxins, coagulase, fibrinolysin, and protein A play significant pathogenic roles, these proteins exhibit high variability among strains or induce non-protective immune responses, making them less suitable for epitope-based vaccine design. Their corresponding amino acid sequences were retrieved from the UniProt.

Table 1. The proteins used to epitope screening

No.	Protein	Gene	UniProt ID	VaxiJen Threshold
1	Foldase protein PrsA	prsA	P60748	0.7676
2	ESAT-6 secretion machinery protein EssA	essA	P0C052	0.7875
3	Penicillin-binding protein PBP2a	mecA	A0A0N9EFF2	0.6032
4	Capsular polysaccharide type 5 biosynthesis protein cap5A	capA	P95695	0.7056
5	Iron-regulated surface determinant protein B	isdB	A6QG30	0.7316
6	Clumping factor A	clfA	Q53653	1.1191
7	Hydroxamate siderophore binding lipoprotein	fhuD2	A0A0H3JRD4	0.6259

2.2. B-Cell epitopes prediction

Linear B-cell epitopes were predicted using the BepiPred-3.0 server available through the DTU Health Tech platform, with a confidence threshold set at 0.5. In addition, conformational epitopes were identified using the IEDB ElliPro tool, selecting those with an exposure score greater than 0.9.

2.3. T-cell epitopes prediction

The binding affinity of antigenic peptides to MHC class I and class II molecules was evaluated using the IEDB T-cell epitope prediction tools. The Stabilized Matrix Method (SMM) was applied to estimate IC_{50} values, with epitopes exhibiting the lowest IC_{50} scores given priority. Additionally, MHC class I immunogenicity was assessed, and only epitopes with scores greater than zero were selected for further analysis.

2.4. Allergenicity

The epitopes identified in the previous stages were screened for allergenic potential using the AllerTOP v.2.0 server, and only non-allergenic candidates were retained for subsequent vaccine construction. The finalized vaccine construct was further evaluated for antigenicity using the VaxiJen v3.0 platform.

2.5. Multi-epitope vaccine sequence construction

To enhance the immunogenicity of the designed vaccine construct, an adjuvant sequence identified as APPHALS was incorporated at the N-terminal region. This addition was intended to reduce potential adverse immune reactions while reinforcing the host's immune defense against the pathogen. Specific peptide linkers—AAY, GPGPG, and EAAAK—were utilized to ensure correct structural organization and efficient epitope presentation. The AAY linker was employed to connect cytotoxic T lymphocyte (CTL) epitopes, while the GPGPG (glycine–proline–glycine–proline) linker was used to join helper T lymphocyte (HTL) and B-cell

epitopes. The EAAAK linker was applied to attach the adjuvant sequence to the main vaccine construct at the N-terminal end. The final designed vaccine sequence is shown below:

APPHALSEAAAKLKDGEVSEVAAYTLGFVIFSIAAYAYEIGIVPKAAYVALIYIFFKA
AYVDKEAFTKAAAYSGDNVIAPVAAYKKDWEETTAGPGPGGYHIIKADKPTDFGPG
PGVIALTFLTASSNNGGPGPGKKMDEYLRDFAKKFGPGPGQKNLKILIILPLLFGPGP
GKQQKEFKSFYSIRKGPGPGSIKVYKVDNAADLGPGPGYAGGLKKLGANIVAGPGP
GKVKSKKSDKGPGPGQQEEEKRINGPGPGQDRKIKKVSGPGPGNDKYSPSKLGPGPG
AEETGGTNTGPGPGSSKEADASEGPGPGQQKLTAKAGGPGPG

2.6. Biochemical characteristic evaluation

An effective vaccine must not only elicit a strong immune response but also exhibit favorable biochemical properties. To evaluate the physicochemical parameters of the designed construct, the ProtParam tool was utilized. This platform estimates several molecular features, including aliphatic index, theoretical isoelectric point (pI), molecular weight, predicted half-life, amino acid composition, instability index, and the grand average of hydropathicity (GRAVY). Protein solubility is a critical factor in vaccine formulation, and multiple computational resources are available for its prediction. The ccSOL omics algorithm was employed to assess solubility based on physicochemical attributes, and the findings were subsequently validated using the Protein-Sol sequence-based solubility database. A solubility value greater than 0.45 was considered indicative of a soluble protein.

2.7. Antigenicity and Allergenicity evaluation of vaccine

In addition to the individual assessment of antigenicity and allergenicity for each epitope, both parameters were also evaluated for the complete vaccine construct. For this purpose, the VaxiJen v3.0 and AllerTOP v2.0 servers were employed, respectively.

2.8. Secondary structure prediction

The secondary structure of a protein defines the folding and spatial arrangement of its polypeptide chain. The principal structural elements include α-helices, β-sheets, β-turns, and random coils. Secondary structure prediction was performed using the Prabi SOPMA server, a widely utilized computational tool for protein structure analysis, applying the default settings for window width and similarity threshold.

2.9. Vaccine tertiary structure prediction

The three-dimensional structure of the designed peptide was generated using the I-TASSER, AlphaFold 18, and Robetta servers. Subsequently, the predicted 3D structures were validated through the VAST tool, and comparative evaluation of the resulting models was conducted using the SAVES v6.0 server. Among the generated models, the structure predicted by Robetta achieved the highest validation scores.

2.10. Molecular docking

Molecular docking analysis plays a pivotal role in elucidating the interaction dynamics and binding affinities between vaccine constructs and host immune receptors, particularly human Toll-like receptor 4 (TLR4). In this study, molecular docking was conducted using the crystal structure of human TLR4 retrieved from the RCSB Protein Data Bank (PDB ID: 2Z63). Additionally, interactions between the designed vaccine construct and selected human leukocyte antigen (HLA) alleles were examined through docking simulations. The accuracy of molecular docking was assessed using Molegro Virtual Docker software, while Molegro Virtual Viewer was employed to visualize the three-dimensional interactions between the vaccine and target proteins.

2.11. Vaccine Structure Construction

The MRSA vaccine construct was generated by cloning the selected antigenic proteins into the **pcDNA3.1(+)** expression vector. To enhance heterologous protein expression efficiency in the murine model, the JCAT and Wrangler tools were utilized. These platforms aided in the optimization of codon usage, as well as the design of prokaryotic ribosome binding sites, transcriptional terminators, and the incorporation of multiple restriction enzyme sites. The gene sequence of the final vaccine construct within the pcDNA3.1(+) vector was validated using the RF-Cloning tool to ensure accurate in-frame expression. Subsequent sequencing of the vaccine constructs was performed by Beijing Corporation (Shenzhen, China).

2.12. Amplification of the Plasmid

The plasmid was introduced into Escherichia coli TOP10F cells using the conventional calcium chloride transformation technique. In this process, bacterial cells were first made competent by treatment with chilled calcium chloride, followed by a brief heat-shock step to temporarily increase membrane permeability and facilitate plasmid entry. The transformed cells were then plated on LB agar containing ampicillin to allow the selective growth of plasmid-bearing bacteria through the expression of the ampicillin resistance gene. After 48 hours of incubation, individual colonies were picked, transferred into fresh LB broth, and cultured to enable large-scale plasmid propagation. The transformation protocol was conducted following Sambrook and Russell (2006). The LB agar medium contained 100 µg/mL ampicillin for selection of recombinant clones (12).

2.13. Verification of Cloning by Enzymatic Digestion

Plasmid DNA was extracted from the transformed Escherichia coli cells using a Favorgen plasmid isolation kit (Taiwan) following the manufacturer's protocol. The quality and integrity of the purified DNA were evaluated by electrophoresis on a 1% agarose gel, and its

concentration was quantified using a Nanodrop spectrophotometer. To verify the presence of the desired gene insert, the plasmid was digested with the restriction enzymes BamHI and EcoRV, which cleave at specific recognition sites flanking the insert region of the vector. Successful digestion confirmed the correct integration of the target sequence. Further validation of the construct was achieved through polymerase chain reaction (PCR) analysis. The PCR conditions included an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute, ending with a final elongation at 72 °C for 5 minutes.

2.14. Administration of the Vaccine Candidate to Animals

Thirty BALB/c mice were procured from the Razi Vaccine and Serum Research Institute in Karaj and housed under standard laboratory conditions. The animals were randomly assigned to two experimental groups—control and vaccinated—each consisting of ten mice. The vaccine candidate was administered intramuscularly into the quadriceps muscle on days 0, 7, and 15. Each injection contained 100 µg of plasmid DNA diluted in 100 µl of phosphate-buffered saline (PBS). Mice in the control group received PBS alone, while those in the vaccinated group were injected with the recombinant plasmid construct.

2.15. Evaluation of gene expression

Total RNA was isolated from mouse spleen tissues using the GeneAll RiboEx Total RNA kit following the manufacturer's protocol. RNA concentration was measured with a NanoDrop 2000 spectrophotometer, and its integrity was verified by electrophoresis on a 1% agarose gel. Complementary DNA (cDNA) was synthesized using a cDNA synthesis kit from Yekta Tajhiz Azma. Quantitative real-time PCR was performed with SYBR Green qPCR Master Mix 2X and gene-specific primers (Table 2). The thermal cycling conditions included an initial

denaturation at 95 °C for 3 minutes, followed by 45 cycles of denaturation at 95 °C for 5 seconds and annealing/extension at 60 °C for 30 seconds. A melting curve analysis was subsequently carried out from 50 °C to 99 °C to confirm the specificity of amplification. Relative gene expression was determined using the 2^−ΔΔCt method.

Table 2. Sequence of primers used in Real time PCR

Gene	Primer Sequence (5'→3')	Product Length (bp)	Ref.
TLR4	F: 5' - ATCATCAGCGTGTCGGTTGTCA- 3' R: 5' - GCAGCCAGCAAGAAGCATCAG- 3'	466	(13)
IL4	F: 5'- ACATCCAGGGAGAGGTTTCCT-3' R: 5'- TGACGCATGTTGAGGAAGAGAC-3'	263	(14)
GAPDH	F: 5′-ACCTTGGAAATAAATGGGAAG-3′ R: 5′- CTTCTGTGTTGCTGTAGTTGC-3′	250	(13)

2.16. Data analysis

Data analysis was carried out using GraphPad Prism software. Statistical differences between groups were assessed by one-way analysis of variance (ANOVA), followed by post hoc evaluation using either the Newman–Keuls multiple comparison test or the Student's t-test. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Epitopes prediction

Epitope selection was performed based on IC_{50} values and corresponding predictive scores, followed by an assessment of their allergenic potential. Table 3 lists the epitopes derived from each protein that exhibited high antigenicity and were predicted to be non-allergenic.

Table 3. Prediction of dominant T and B cell epitopes of each protein

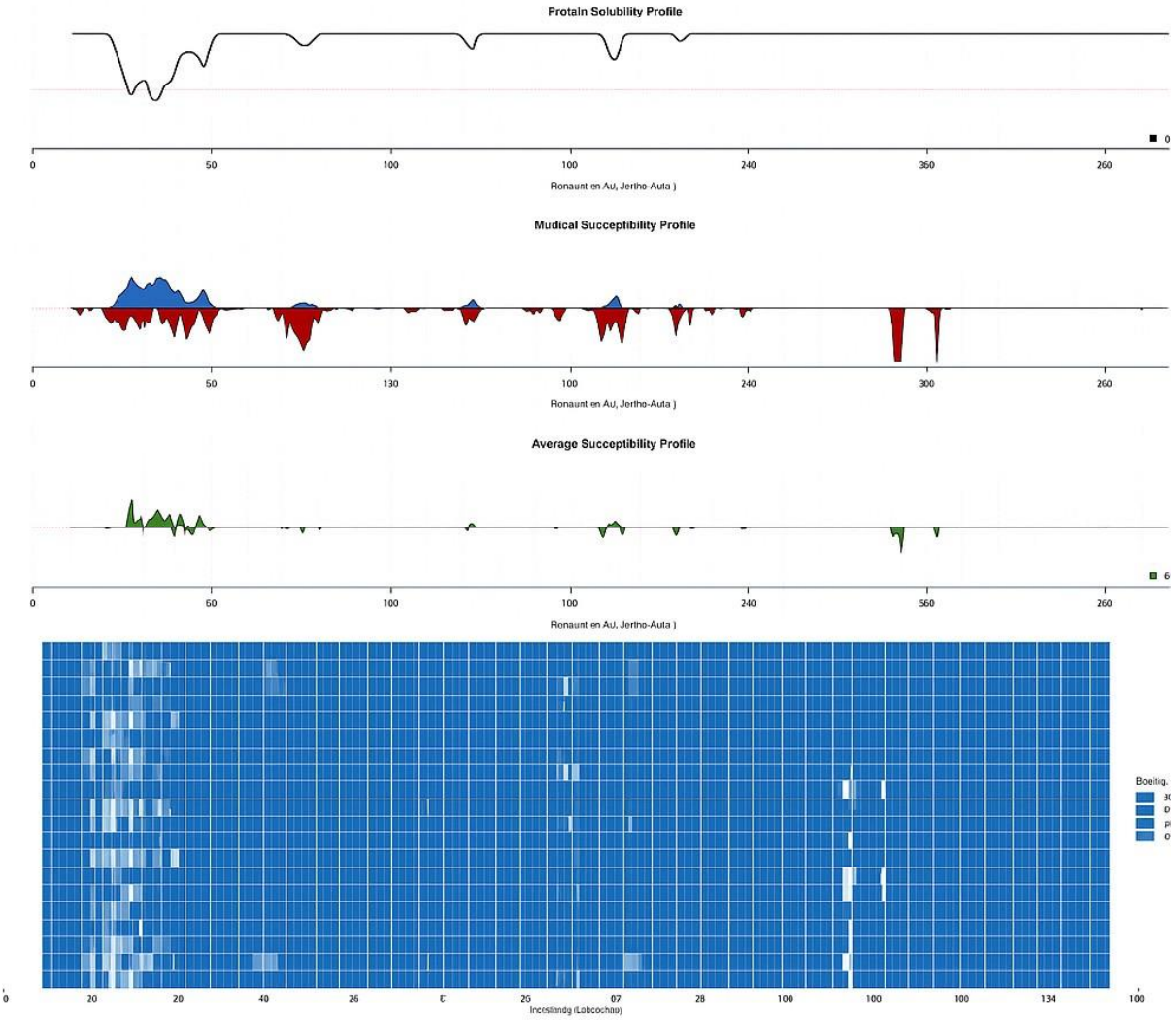
Protein	Epitope	Sequence
PrsA	CTL	LKDGEVSEV
	HTL	GYHIIKADKPTDF
	Linear B cell	KVKSKKSDK
EssA	CTL	TLGFVIFSI
	HTL	VIALTFLTASSNNG
	Linear B cell	QQEEEKRIN
PBP2a	CTL	AYEIGIVPK
	HTL	KKMDEYLRDFAKKF
	Linear B cell	QDRKIKKVS
Cap5A	CTL	VALIYIFFK
	HTL	QKNLKILIILPLLF
	Linear B cell	NDKYSPSKL
IsdB	CTL	VDKEAFTKA
	HTL	KQQKEFKSFYSIRK
	Linear B cell	AEETGGTNT
ClfA	CTL	SGDNVIAPV
	HTL	SIKVYKVDNAADL
	Linear B cell	SSKEADASE
FhuD2	CTL	KKDWEETTA
	HTL	YAGGLKKLGANIVA
	Linear B cell	QQKLTAKAG

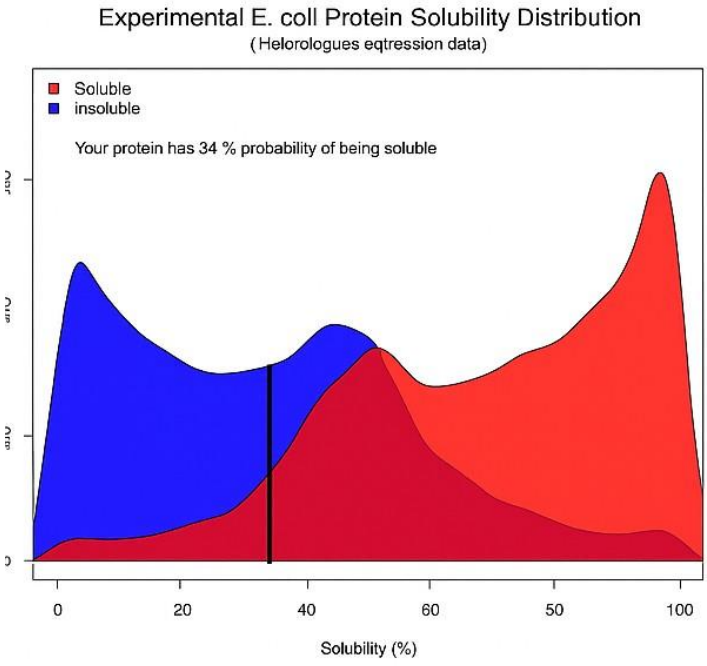
3.2. Vaccine sequence construction

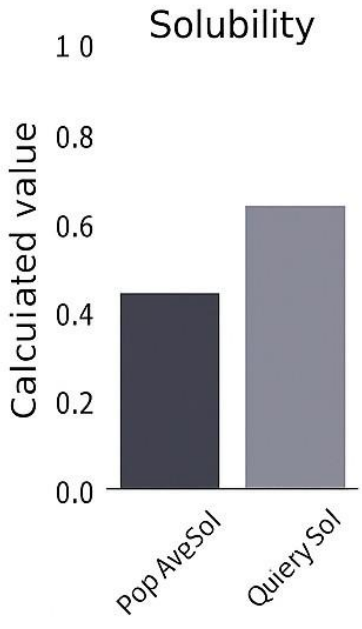
After merging overlapping sequences, suitable peptide linkers were used to assemble the selected epitopes. The resulting multi-epitope vaccine construct consisted of four main domains: an adjuvant, B-cell epitopes, cytotoxic T lymphocyte (CTL) epitopes, and helper T lymphocyte (HTL) epitopes. To enhance structural stability and prevent degradation, the APPHALS adjuvant was positioned at the N-terminal end of the construct and linked to the CTL epitopes through an EAAAK linker. Furthermore, AAY and GPGPG linkers were employed to connect CTL to HTL epitopes and to join B-cell epitopes, respectively.

3.3. Evaluation of physical and chemical properties

An online computational platform was employed to assess the physicochemical characteristics, immunogenic potential, and allergenicity of the designed multi-epitope vaccine. Analysis using ProtParam revealed that the construct comprises 327 amino acids, with a molecular weight of 33.30 kDa and a theoretical isoelectric point (pI) of 9.43. The predicted half-life was estimated to be 14.4 hours in mammalian reticulocytes, over 20 hours in yeast, and more than 10 hours in *Escherichia coli*. The calculated instability index of 22.60 indicated that the vaccine construct is stable. The aliphatic index and GRAVY (grand average of hydropathicity) values were 65.47 and −0.468, respectively. As shown in Figure 1, the solubility of the vaccine construct was predicted to be 34%. According to VaxiJen v3.0 analysis, the construct was classified as a probable antigen with 100% probability. In addition, predictions from the AllerTOP v2.0 server identified the designed multi-epitope vaccine as non-allergenic.


Protain Solubility Profile
Ronaunt en Au, Jertho-Auta)
Mudical Succeptibility Profile
Ronaunt en Au, Jertho-Auta)
Average Succeptibility Profile
Ronaunt en Au, Jertho-Auta)


Experimental E. coll Protein Solubility Distribution
(Helorologues eqtression data)
Soluble
insoluble
Your protein has 34 % probability of being soluble
Solubility (%)


Solubility
Calcuiated value
Pop AveSol
Quiery Sol

280

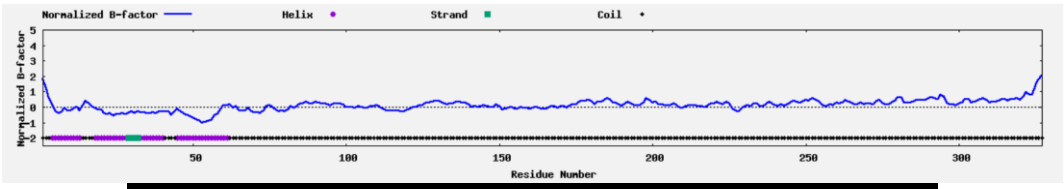
Figure 1. Predicted solubility of the vaccine construct using the ccSOL server. The scaled solubility value (QuerySol) represents the predicted solubility of the construct. The population average solubility value for the experimental dataset (PopAvrSol) is 0.45; thus, any scaled solubility value above 0.45 indicates a higher solubility compared to the average soluble *Escherichia coli* protein in the experimental solubility dataset reported by Niwa et al. (2009), while a lower value suggests reduced solubility (15).

3.4. Secondary structure prediction

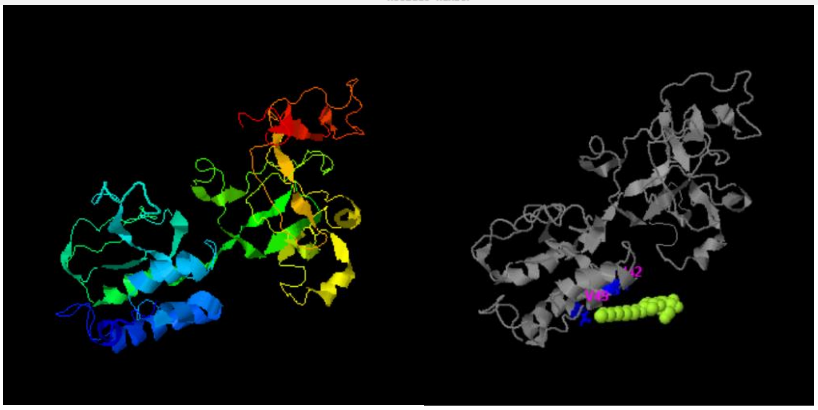
The secondary structure of the designed multi-epitope vaccine was predicted using the Prabi server. The analysis indicated that the construct consists of 25.38% α-helices, 19.57% extended β-strands, and 55.05% random coils.

3.5. Modeling and refining the tertiary structure of the vaccine

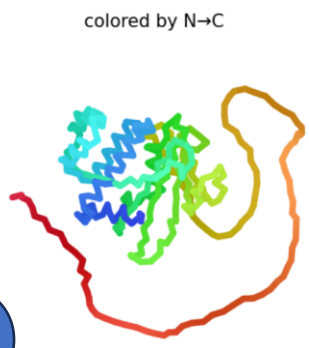
The tertiary structure of the multi-epitope vaccine was modeled using the I-TASSER, AlphaFold, and Robetta servers. In the I-TASSER analysis, Model 4 (Figure 2A) was identified as the optimized structure, with a C-score of −4.62, an estimated TM-score of 0.38 ± 0.13, and an RMSD of 13.5 ± 4.0 Å. From the AlphaFold predictions, Model 3 (Figure 2B) was selected based on its pLDDT value (31.7), pTM score (0.205), and tolerance (5.66). The structure predicted by Robetta is shown in Figure 2C. A comparative assessment of the three generated models using the SAVES server revealed that the Robetta-derived model demonstrated the highest overall quality and was therefore chosen for further structural analyses (Figure 2C).


Normalized B-factor
Helix
Strand
Coil
Residue Number

A



B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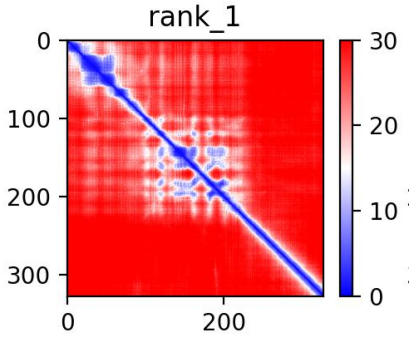
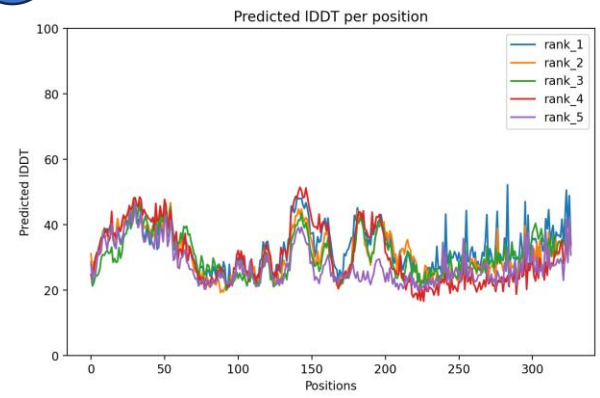

rank_1


Predicted IDDT per position
rank_1
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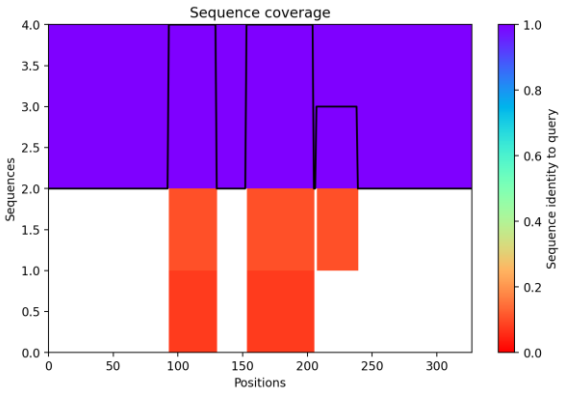
Predicted IDDT
Positions


Sequence coverage
Sequences
Positions
Sequence identity to query

C

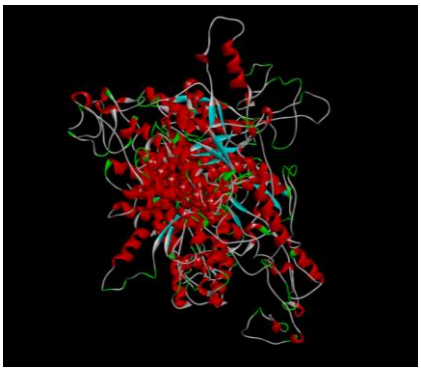


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Figure 2. (A) Tertiary structure of the vaccine predicted using the I-TASSER server: (1) predicted normalized B-factor, (2) final 3D model generated by I-TASSER, and (3) predicted ligand-binding sites. (B) Tertiary structure of the vaccine obtained from the AlphaFold database. (C) Tertiary structure of the vaccine model generated using the Robetta server.

3.6. Molecular docking

The molecular docking outcomes are illustrated in Figure 3. Docking simulations were conducted between the designed vaccine construct and all target proteins, resulting in binding energy values that fell within an acceptable and biologically relevant range.

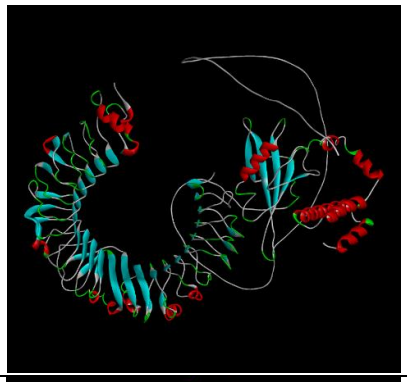
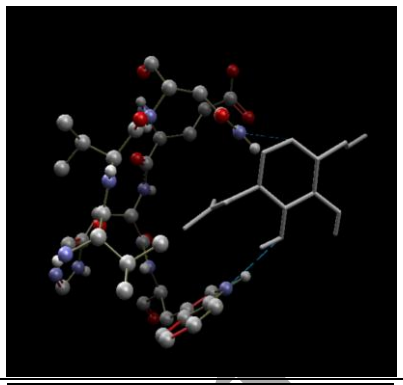
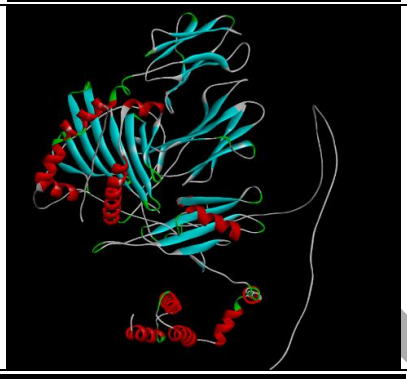
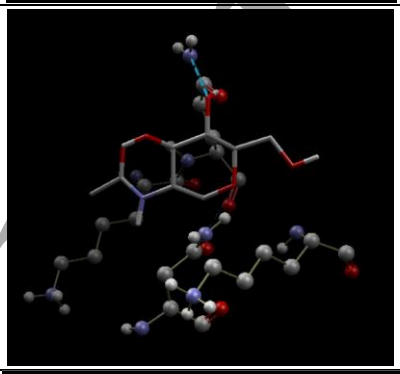
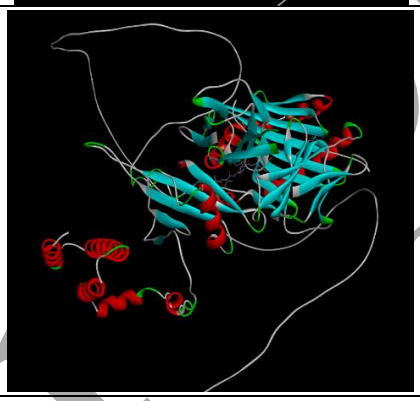
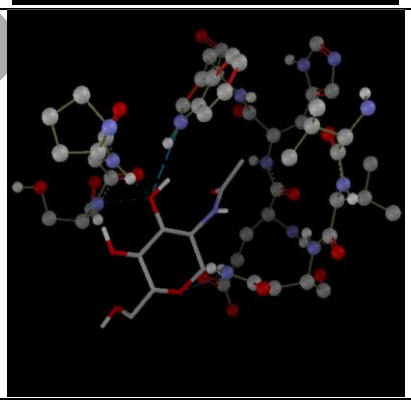
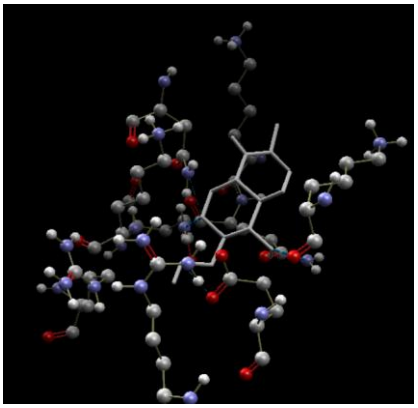
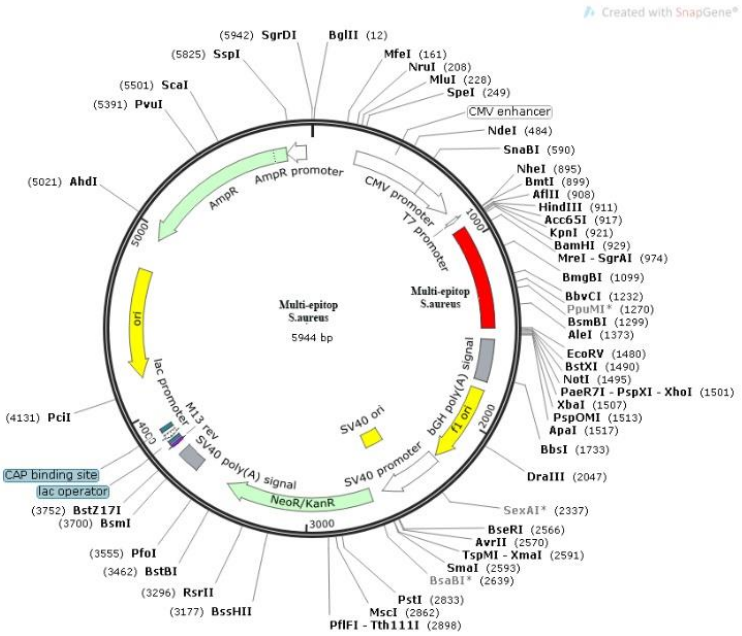
Receptor (PDB ID) Docking energy	Molecular Docking view	Atoms Interaction
TLR4 (2z63) -78.23 kJ/mol		
HLADR B1_03:01 (1A6A) -32.86 kJ/mol		
HLA-DR B3_02:02 (3C5J) -60.74 kJ/mol		
HLADRB1_01:01 (1AQD) -61.24 kJ/mol		

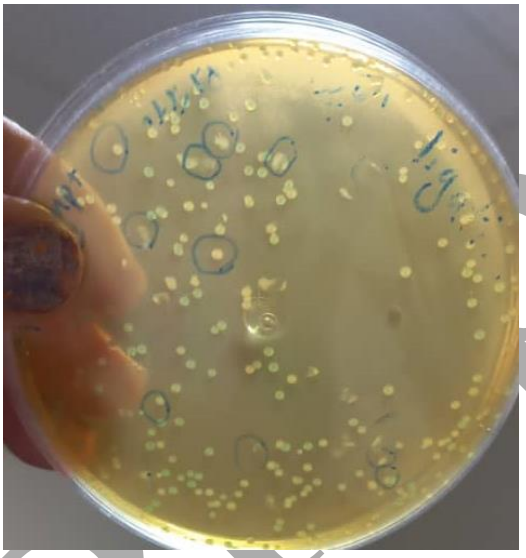
Figure 3. Molecular docking analysis of the vaccine construct with TLR4 and selected HLA alleles. The left column displays the receptor molecule and the ligand (vaccine), while the right column illustrates atomic-level interactions. In the visualization, the ball-and-stick model represents the receptor, and the gray linear model corresponds to the ligand (vaccine). Intermolecular interactions are indicated by blue dashed lines.

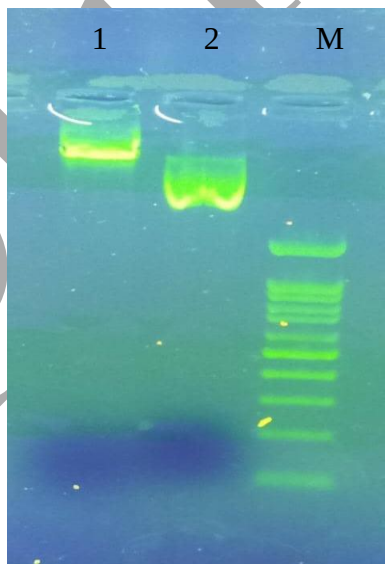
3.7. Codon optimization of the designed vaccine

The vaccine construct was optimized for expression in *Escherichia coli* using the JCAT tool, followed by codon optimization to improve expression efficiency in the murine host. The optimization process resulted in a Codon Adaptation Index (CAI) of 0.9, indicating a strong likelihood of successful expression, as CAI values above 0.80 are generally considered optimal for the chosen host system. The optimized sequence displayed a GC content of 64.37%, further supporting its suitability for expression. The integrity of the dual-vaccine sequence within the pcDNA3.1 expression vector was confirmed using the restriction enzymes BamHI and EcoRV, with analysis conducted via the GenScript server. Recognition sites for BamHI (GGA TCC) and EcoRV (GAT ATC) were inserted at the 5′ and 3′ ends of the construct, respectively. Finally, the dual-vaccine construct was modeled *in silico* using the Addgene platform to verify accurate insertion and expression within the pcDNA3.1 vector at the designated restriction sites (Figure 4A).

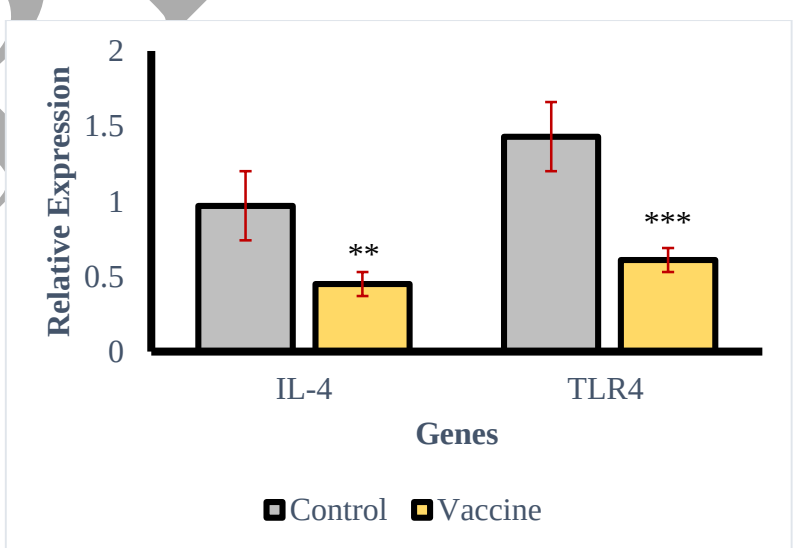

Created with SnapGene®
(5942) SgrDI
BglII (12)
(5825) SspI
MfeI (161)
NruI (208)
MluI (228)
SpeI (249)
(5501) ScaI
(5391) PvuI
CMV enhancer
NdeI (484)
SnaBI (590)
(5021) AhdI
NheI (895)
BmtI (899)
AflII (908)
HindIII (911)
Acc65I (917)
KpnI (921)
BamHI (929)
MreI - SgrAI (974)
BmgBI (1099)
BbvCI (1232)
PpuMI* (1270)
BsmBI (1299)
AleI (1373)
EcoRV (1480)
BstXI (1490)
NotI (1495)
PaeR7I - PspXI - XhoI (1501)
XbaI (1507)
PspOMI (1513)
ApaI (1517)
BbsI (1733)
DraIII (2047)
(4131) PciI
CAP binding site
lac operator
(3752) BstZ17I
(3700) BsmI
SexAI* (2337)
BseRI (2566)
AvrII (2570)
TspMI - XmaI (2591)
SmaI (2593)
BsaBI* (2639)
(3555) PfoI
(3462) BstBI
(3296) RsrII
(3177) BssHII
PstI (2833)
MscI (2862)
PflFI - Tth111I (2898)
AmpR
AmpR promoter
CMV promoter
T7 promoter
Multi-epitop S.aureus
Multi-epitop S.aureus
5944 bp
ori
lac promoter
M13 rev
SV40 poly(A) signal
SV40 ori
bGH poly(A) signal
f1 ori
SV40 promoter
NeoR/KanR
1000
2000
3000
4000
5000

A




1
2
M

B


Relative Expression
2
1.5
1
0.5
0
**
***
IL-4
TLR4
Genes
Control
Vaccine

C

Figure 4. (A) The designed vaccine construct cloned into the pcDNA3.1 expression vector. (B) Verification of successful cloning. (1) Ligation product grown in an ampicillin-containing medium; (2) results of enzymatic digestion (lane 1: recombinant plasmid, lane 2: empty plasmid, M: molecular marker); (3) analysis of IL-4 and TLR4 gene expression levels in mouse spleen tissue following vaccination (P < 0.01, ***P < 0.001).**

3.8. Cloning assessment

The successful growth of transformed bacteria on penicillin-containing medium confirmed the presence of the plasmid carrying the penicillin resistance gene (Figure 4B). To ensure the correct insertion of the multi-epitope gene into *Escherichia coli* TOP10F cells, restriction enzyme digestion was carried out. The plasmid concentration was determined spectrophotometrically, and its integrity was evaluated using agarose gel electrophoresis. The digestion results confirmed that the target gene fragment had been accurately inserted into the plasmid construct (Figure 4B).

3.9. Gene expression analysis

Analysis of TLR4 and IL-4 gene expression demonstrated a statistically significant difference between the control group and the group immunized with the vaccine candidate ($P < 0.05$). Both genes showed notable downregulation in the spleen tissues of vaccinated mice compared with those of the control group (Figure 4C).

4. Discussion

In light of the ongoing emergence of multidrug-resistant bacterial strains, coupled with the high costs, prolonged development timelines, and limited effectiveness of conventional antibiotic therapies, substantial research efforts have focused on the development of an effective vaccine against *Staphylococcus aureus*. Nevertheless, despite more than two decades of intensive investigation, no commercially licensed vaccine is currently available for this pathogen. In this

study, a rationally designed multi-epitope vaccine targeting *S. aureus* was developed using seven virulence-associated proteins as antigenic sources. The predicted epitopes were systematically assessed for their antigenic and allergenic properties, and those exhibiting the highest immunogenic potential were selected for vaccine construction. The chosen epitopes were connected using specific peptide linkers, and the APPHALS adjuvant was strategically fused to the N-terminal region to improve structural stability and receptor-binding affinity. The physicochemical properties of the final vaccine construct were evaluated using the ProtParam and ccSOL servers, while secondary and tertiary structural predictions were performed using the Prabi, AlphaFold, I-TASSER, and Robetta platforms. The overall antigenicity and allergenicity of the assembled construct were subsequently confirmed using the VaxiJen and AllerTOP tools, respectively. Structural validation followed by molecular docking analyses with TLR4 and representative HLA alleles, performed using Molegro Virtual Docker, demonstrated strong and stable interactions between the designed vaccine construct and immune cell surface receptors. Despite exhibiting high antigenicity, the construct showed no allergenic potential. After cloning the candidate sequence into the pcDNA3.1(+) expression vector and confirming recombinant expression, the immunomodulatory effects of the vaccine were evaluated by measuring TLR4 and IL-4 gene expression levels in murine spleen tissues. In agreement with the docking predictions, both genes were significantly downregulated in the vaccinated group, supporting the proposed construct as a promising and safe vaccine candidate against *Staphylococcus aureus* infection. Moreover, although the present study was limited to transcriptional analysis of immune markers (TLR4 and IL-4), future investigations will focus on evaluating cytokine secretion profiles, antibody titers, and T-cell proliferation to provide a more comprehensive assessment of the elicited immune protection. PrsA belongs to the parvulin-type peptidyl-prolyl cis/trans isomerase (PPIase) family and is predominantly associated with Gram-positive bacteria, where it functions as a molecular chaperone involved

in proper protein folding. In *Staphylococcus aureus*, as in other Gram-positive organisms, PrsA is expressed but is not essential for bacterial survival (16). In addition to its function as a molecular chaperone, PrsA is involved in membrane lipid remodeling and contributes to the decreased susceptibility of *Staphylococcus aureus* to β-lactam antibiotics through its functional association with daptomycin. Furthermore, PrsA is a surface-exposed protein with strong antigenic properties, making it readily detectable by the host immune system. Due to its dual role as both a surface antigen and a regulator of virulence-related processes, PrsA constitutes an attractive target for the development of effective vaccines against *S. aureus* (17). This protein induces a robust antibody-mediated immune response and provides long-lasting protection against multiple infections in murine models. In Streptococcus sanguinis, PrsA has been recognized as a promising vaccine antigen due to its capacity to stimulate the production of opsonic antibodies. Additionally, PrsA is highly conserved in Legionella pneumophila and displays strong immunogenicity, further reinforcing its potential as a target for DNA-based vaccine development (18). The results of the present study suggest that PrsA derived from MRSA has the potential to induce cross-protective immunity against diverse pathogenic *Staphylococcus aureus* strains, thereby representing a novel and promising antigenic candidate for the development of an effective MRSA vaccine.

The findings of this study indicate that MRSA-derived PrsA may stimulate cross-protective immune responses against a broad range of pathogenic *Staphylococcus aureus* strains, highlighting its potential as a novel and promising antigen for the development of an effective MRSA vaccine (19). EssA, an immune-protective antigen, shares a high degree of sequence homology with a corresponding protein from Mycobacterium tuberculosis and exhibits significant virulence-associated and immunogenic characteristics. Moreover, EssA has been reported to elicit a potent immune response against Streptococcus agalactiae infection (18). Previous studies have demonstrated that highly antigenic proteins are readily recognized by the

406 host immune system and are capable of eliciting potent immune responses. Among these, EssA
407 has been identified as a particularly promising vaccine candidate. Furthermore, the integration
408 of multiple epitopes derived from such immunogenic proteins—each capable of activating
409 distinct immune pathways—may facilitate the development of a broad-spectrum and
410 potentially universal vaccine against *S. aureus* (6). Studies have shown that penicillin-binding
411 protein 2A (PBP2A), a central determinant of resistance in MRSA, enables *Staphylococcus*
412 *aureus* to withstand exposure to β-lactam antibiotics, thereby contributing significantly to
413 infection-associated morbidity and mortality worldwide. Previous investigations using
414 antibodies targeting PBP2A demonstrated no cross-reactivity with methicillin-sensitive *S.*
415 *aureus* (MSSA), underscoring the high specificity of anti-PBP2A antibodies for MRSA strains
416 (20, 21).

417 The capsular polysaccharides (CPs) of *S. aureus* play a critical role in immune evasion by
418 inhibiting complement activation and preventing neutrophil-mediated phagocytic clearance.
419 CPs have been effectively exploited as target antigens in several licensed bacterial vaccines,
420 including those developed against *Haemophilus influenzae* type b, *Streptococcus pneumoniae*,
421 and *Neisseria meningitidis* serogroups A, C, W, and Y (22, 23). Building on the success of
422 polysaccharide-based vaccines developed for other bacterial pathogens, several clinical trials
423 have been initiated to evaluate *S. aureus* vaccine candidates derived from capsular
424 polysaccharides. To enhance the immunogenicity of CPs and promote T-cell memory
425 responses, the *S. aureus* CP5 and CP8 polysaccharides were conjugated to carrier proteins for
426 assessment in preclinical and early-phase clinical studies (24). Preclinical studies employing
427 CP5- and CP8-specific antibodies or CP-conjugated vaccines demonstrated partial protection
428 in animal models of *S. aureus* infection. Moreover, polyclonal antibodies directed against
429 staphylococcal capsular polysaccharides were shown to enhance the phagocytic clearance of
430 encapsulated *S. aureus* cells. StaphVax, developed by Nabi Biopharmaceuticals, represented

the first-generation vaccine composed of CP5 and CP8 polysaccharides conjugated to a detoxified recombinant form of Pseudomonas aeruginosa exotoxin A. Although StaphVax was the first *S. aureus* vaccine candidate to advance to phase III clinical trials, it ultimately failed to demonstrate efficacy in patients with end-stage renal disease (25). Concerns regarding the efficacy of StaphVax arose from observations that certain *S. aureus* isolates expressed low levels of capsular polysaccharides and that the targeted patient population may have been unable to mount sufficiently protective immune responses. Moreover, the vaccine failed to consistently induce effective antibacterial activity within this specific cohort of patients (26).

The srtB and isdC genes belong to the isd locus, which also encodes the sortase A-anchored proteins IsdA and IsdB, the membrane transport complex IsdEF, and the cytoplasmic protein IsdG. IsdB is highly conserved among a wide range of *S. aureus* clinical isolates, including both methicillin-resistant and methicillin-sensitive strains. The detection of surface-expressed IsdB in bacteria grown in vivo, together with experimental evidence supporting its protective role, suggests that this protein is rapidly upregulated during infection. Moreover, elevated anti-IsdB antibody titers have been directly correlated with improved survival in murine sepsis models. Consequently, an IsdB-based vaccine represents a promising antigenic candidate for preventing *S. aureus* infections by boosting preexisting antibody levels to protective thresholds capable of conferring effective immunity (27).

Two structurally related fibrinogen-binding proteins, known as clumping factors (ClfA and ClfB), are expressed on the surface of *S. aureus* and play a pivotal role in its virulence and pathogenic potential (28). A previous study demonstrated that mice intranasally immunized with ClfA prior to exposure to a sublethal dose of *S. aureus* exhibited a significant reduction in bacterial burden both systemically (in the kidneys and spleen) and locally at the infection site (peritoneal cavity) throughout the course of infection. In addition, vaccinated mice showed

enhanced recruitment of neutrophils and macrophages, accompanied by increased T-cell activation, compared with non-immunized controls. The staphylococcal ClfA protein has been recognized as a promising component for inclusion in multivalent vaccine formulations, as it is widely expressed among clinical isolates and capable of inducing robust T-cell responses and interferon-gamma (IFN-γ) secretion when co-administered with heat-killed *S. aureus*. Moreover, ClfA functions as a key virulence determinant, and multiple studies have confirmed its protective efficacy across diverse experimental models of staphylococcal infection (29).

FhuD2 belongs to a family of predicted iron-binding proteins that are widely distributed among Gram-positive bacteria. In *S. aureus*, the ABC transporter complex composed of FhuCBG, FhuD1, and FhuD2 mediates the uptake of iron(III)-hydroxamates. FhuD2 interacts with multiple types of iron(III)-hydroxamates with varying binding affinities, exhibiting particularly strong affinity for iron(III)-ferrichrome and iron(III)-desferrioxamine (30).

Conclusion

Vaccination offers a promising approach to counteract MRSA infections amid the rising challenge of antibiotic resistance. In this study, a multi-epitope vaccine was designed and evaluated using key proteins implicated in MRSA pathogenesis. The findings revealed that the constructed vaccine exhibited effective interactions with immune cell surface receptors, suggesting strong potential for specific target binding. Bioinformatics offers a robust platform for the rational design of therapeutic agents, including vaccines. Nevertheless, a complete evaluation of vaccine efficacy necessitates experimental validation through synthesis and subsequent detailed in vitro and in vivo studies.

Declarations

Ethical Approval: This study was not on animal or human samples and did not require ethical approval.

Consent to Participate: This study was not on animal or human samples and did not require consent to participate.

Consent to Publish: This study was not on animal or human samples and did not require consent to publish.

Author Contribution: S.M.M designed research; performed research; contributed analytic tools; analyzed data; and reviewed the paper. M.S, A.H., B.S.T, R.A., and AI helped in designing the research, analyzed data, and wrote the paper.

Funding: This study had no funding.

Competing Interests: The authors declare no competing interests.

Availability of data and materials: All data is available in the manuscript.

Acknowledgements: The authors declare that the language of this manuscript was refined using GPT-5 (OpenAI, 2025), an artificial intelligence language model, for paraphrasing and clarity improvement.

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