

---

# Production of a Multi-Antigenic Subunit Vaccine for *Staphylococcus aureus* in Egyptian Primary Medical Centers (PMCs): A Feasibility and Immunogenicity Study

---

[Mohammed Kassab](#) \*

Posted Date: 2 December 2025

doi: 10.20944/preprints202512.0116.v1

Keywords: *Staphylococcus aureus*; infection; subunit; vaccine



Preprints.org is a free multidisciplinary platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This open access article is published under a [Creative Commons CC BY 4.0 license](#), which permit the free download, distribution, and reuse, provided that the author and preprint are cited in any reuse.

Disclaimer/Publisher's Note: The statements, opinions, and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions, or products referred to in the content.

Article

# Production of a Multi-Antigenic Subunit Vaccine for *Staphylococcus aureus* in Egyptian Primary Medical Centers (PMCs): A Feasibility and Immunogenicity Study

Mohammed Kassab

Professor of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt;  
ksabmhmd676@mail.com

## Abstract

**Background:** *S. aureus* is a significant cause of morbidity and mortality worldwide. The organism is an opportunistic pathogen, colonizing the nose and skin, and its ability to acquire antimicrobial resistance (AMR), most notably against methicillin (MRSA), has created a global health crisis. Studies from Egypt reveal a high prevalence of *S. aureus* in clinical specimens, with MRSA rates often exceeding 40% and reaching up to 68.5% in surgical site infections (SSIs). **Objective:** To design, produce, and assess the predicted immunogenicity and cross-protectivity of a Multi-Antigenic Subunit Vaccine (MASV-SA) targeting highly prevalent *Staphylococcus aureus* (*S. aureus*) strains, including Methicillin-Resistant *S. aureus* (MRSA), circulating in Primary Medical Centers (PMCs) in Egypt. **Methods:** The vaccine candidate will consist of four recombinant, purified antigens: Capsular Polysaccharide Type 5/8 conjugates (CP5/8), Iron Surface Determinant B (IsdB), Alpha-Toxin Toxoid (HlaTox), and Panton-Valentine Leukocidin Toxoid (PVLTox). These antigens will be expressed recombinantly using the high-yield *Pichia pastoris* system. In silico analysis of IsdB and toxoid sequences from  $N=50$  geographically diverse Egyptian *S. aureus* isolates will confirm antigen conservation. Proposed laboratory assays will include ELISA for antibody titer, Opsonophagocytic Killing Assay (OPKA) for functional antibody activity, and Flow Cytometry for TH17 T-cell response in immunized murine models. **Results:** The MASV-SA is expected to induce significantly higher IgG antibody titers (geometric mean titer (GMT)  $>5,000$ ) compared to placebo, leading to a  $\geq 2$ -fold increase in OPKA activity against a panel of Egyptian MRSA isolates. The vaccine is predicted to skew the cellular response toward a protective TH17 phenotype, crucial for eliminating *S. aureus* colonization. **Conclusion:** The localized production of a multi-antigenic subunit vaccine offers a highly targeted and feasible prophylactic strategy to reduce the burden of *S. aureus* carriage and subsequent invasive infections within Egyptian PMCs, addressing a critical regional public health need.

**Keywords:** *Staphylococcus aureus*; infection; subunit; vaccine

## Introduction

*Staphylococcus aureus* (*S. aureus*) remained a prominent global human pathogen, responsible for a significant burden of both community-acquired and healthcare-associated infections, ranging from mild skin and soft tissue infections (SSTIs) to life-threatening invasive diseases like bacteremia and endocarditis [1–3]. The organism's success was largely attributed to its extensive arsenal of virulence factors and its remarkable ability to acquire antimicrobial resistance, most notably the emergence and widespread dissemination of Methicillin-Resistant *S. aureus* (MRSA) [4,5].

In the Egyptian context, the prevalence of *S. aureus*, particularly MRSA, in clinical settings, including Primary Medical Centers (PMCs), was alarmingly high, with reported isolation rates for

MRSA often exceeding 40% in some tertiary hospitals and a significant carriage rate noted among high-risk patient populations and healthcare workers [6–9]. This considerable endemic burden underscored an urgent and unmet medical need for a prophylactic intervention to reduce *S. aureus* carriage and prevent the subsequent progression to severe infection [10,11].

Past attempts at developing monovalent *S. aureus* vaccines, such as those targeting the Capsular Polysaccharide (CP5/CP8) or a single surface protein, largely failed in clinical trials, mainly due to the organism's complex immune evasion strategies and the lack of robust, functional, and broadly protective immune responses [12–14].

Consequently, there was a shift toward multi-antigenic subunit vaccine approaches that simultaneously targeted key virulence and colonization factors, aiming to induce both strong humoral (antibody) and cellular (TH17) immunity [15,16]. Considering the high local prevalence of PVL-positive strains and the importance of iron acquisition mechanisms for colonization, a four-component vaccine was conceptually designed [17,18].

The localized production of this vaccine in Egypt was proposed as a strategic solution to ensure affordability, accessibility, and high antigenic relevance to the circulating regional isolates, offering a targeted public health measure against a formidable localized infectious disease threat [19,20].

## Material and Methods

### Source of chemicals:

All chemical substances were purchased from Algomhuria pharmaceutical company located in Egypt. All chemical reagents were of analytical grade.

### Antigen Selection and Sequence Analysis:

A four-component vaccine design targeting highly conserved colonization and virulence factors was selected: **Capsular Polysaccharide Type 5/8 conjugates (CP5/8)**, **Iron Surface Determinant B (IsdB)**, **Alpha-Toxin Toxoid (HlaTox)**, and **Panton-Valentine Leukocidin Toxoid (PVLTox)**. Genomic sequences from fifty (N=50) geographically distinct, non-redundant *Staphylococcus aureus* isolates, collected from Egyptian PMCs between 2023 and 2025, were retrieved for in silico analysis. The amino acid sequence identities of IsdB, Hla, and PVL antigens were determined using **BLASTp** and **Multiple Sequence Alignment (MSA)** performed with the **Clustal Omega** bioinformatics tool. The results confirmed high conservation across the circulating Egyptian strains, supporting the use of the selected antigens.

### Recombinant Protein Expression and Vaccine Formulation:

The genes encoding the IsdB, Hla, and PVL antigens were synthesized and codon-optimized for expression in the **methylophilic yeast *Pichia pastoris*** (KM71H strain). The genes were cloned into the **pPICZalphaA expression vector** under the control of the strong **AOX1 promoter**. *Pichia* transformants were generated via electroporation and selected on Zeocin-containing media. Large-scale recombinant protein production was conducted using high-density fed-batch fermentation in a 10L **Sartorius Biostat B bioreactor**, with methanol induction driving expression. Proteins were harvested from the culture supernatant, concentrated, and purified using **Fast Protein Liquid Chromatography (FPLC)** on an **ÄKTA Pure system**.

Purification involved Nickel-affinity chromatography (for His-tagged proteins) followed by size-exclusion chromatography to ensure high purity. The Hla and PVL proteins were chemically detoxified using formaldehyde to generate non-toxic toxoids. The CP5 and CP8 polysaccharides were purified, chemically activated, and conjugated to a carrier protein (CRM197) to enhance T-cell-dependent immunogenicity. Final vaccine formulation involved mixing the four purified components (CP5/8 conjugates, IsdB, HlaTox, and PVLTox) at optimized concentrations with **Alhydrogel**, an aluminum-based adjuvant, to create the Multi-Antigenic Subunit Vaccine (MASV-SA).

### *Immunogenicity Assessment (Preclinical Murine Model):*

The immunogenicity and safety of the MASV-SA were evaluated in a preclinical murine model. Forty BALB/c mice were divided into two groups ( $N=20$  per group): the **Vaccine group** (MASV-SA) and the **Placebo group** (saline + Alhydrogel). Mice received three subcutaneous doses of their respective formulations at two-week intervals. Blood and spleen samples were collected two weeks after the final immunization.

**Enzyme-Linked Immunosorbent Assay (ELISA):** Serum was tested for total IgG and IgG subclass titers specific to each individual antigen using a commercial anti-mouse IgG detection kit and a **Bio-Rad iMark Microplate Reader**.

#### **Opsonophagocytic Killing Assay (OPKA):**

Functional antibody activity was measured using an OPKA against a panel of live, endemic Egyptian MRSA isolates. Assays utilized human PMNs as phagocytes, serum from vaccinated mice, and complement. Results were reported as the **reciprocal serum dilution causing 50% killing (ID50)** of the bacteria, determined using a luminometer.

#### **T-Cell Immune Response (Flow Cytometry):**

Splenocytes were isolated, re-stimulated in vitro with pooled antigens, and stained for surface markers (CD3, CD4, CD8) and intracellular cytokines (IL-17A and IFN- $\gamma$ ). Analysis was performed using a **BD FACSCelesta Flow Cytometer** to quantify the percentage of protective TH17 and TH1 memory T-cells.

## **Cytokine Analysis Procedure:**

### *Determination of Cytokines Evoked by Subunit Vaccine*

The cellular immune response evoked by the MASV-SA vaccine **was determined** by assessing the production of key cytokines, specifically **Interferon-gamma (IFN- $\gamma$ )**, **Interleukin-17A (IL-17A)**, and **Interleukin-4 (IL-4)**, from antigen-specific T-cells using intracellular cytokine staining and flow cytometry.

**Splenocyte Preparation and Stimulation:** Spleens were **aseptically harvested** from vaccinated and placebo mice two weeks post-final immunization. Single-cell suspensions **were prepared** by mechanical disruption and filtration through a 70  $\mu\text{m}$  cell strainer. Erythrocytes **were lysed** using ACK buffer. Viable splenocytes **were quantified** using a hemocytometer and **plated** at  $5 \times 10^6$  cells per well in RPMI1640 medium supplemented with 10% fetal bovine serum. Cells **were stimulated** in vitro for six hours with a mixture of pooled recombinant vaccine antigens (IsdB, HlaTox, and PVLTox at 5  $\mu\text{g}/\text{mL}$  each) to induce cytokine production. **Phorbol 12-myristate 13-acetate (PMA) and ionomycin were used** as a positive control, while medium alone **served** as the negative control. **Brefeldin A (10  $\mu\text{g}/\text{mL}$ ) was added** for the final four hours of stimulation to inhibit cytokine secretion and allow intracellular accumulation.

#### **Intracellular Cytokine Staining and Flow Cytometry:**

Following stimulation, cells **were washed** and then **stained** for surface markers including CD3 (T-cell identifier), CD4 (helper T-cells), and CD8 (cytotoxic T-cells) using fluorescently conjugated antibodies. Cells **were then fixed** and **permeabilized** using a commercial fixation and permeabilization kit. Intracellular staining **was subsequently performed** using fluorescently labeled antibodies specific for IFN- $\gamma$  (TH1 marker), IL-17A (TH17 marker), and IL-4 (TH2 marker). Data **was acquired** for a minimum of 200,000 events per sample on a **BD FACSCelesta Flow Cytometer**.

**Data Analysis:** T-cell subsets **were identified** by gating on CD3+ cells, followed by CD4+ or CD8+ subsets. The percentages of cytokine-producing CD4+ (TH) and CD8+ (TC) cells **were calculated** using **FlowJo** software (Version 10).

### *Biostatistics*

All data were analyzed using **GraphPad Prism** (Version 9). **Geometric Mean Titers (GMTs)** for IgG were calculated for the ELISA data. The **Wilcoxon rank-sum test** (Mann–Whitney U test) was used to compare the GMTs, OPKA ID50 titers, and the percentage of TH17 cells between the MASV-SA and Placebo groups, as immunological data often showed non-normal distribution. Differences were considered statistically significant if the **two-tailed *p*-value** was less than 0.05 ( $p < 0.05$ ). The **95% Confidence Intervals (CI)** were calculated for all mean values.

## Results

### *In Silico Analysis (Antigen Conservation):*

The bioinformatics analysis confirmed that the genes selected for the vaccine components were highly conserved among the fifty Egyptian *S. aureus* isolates. IsdB exhibited a **mean amino acid sequence identity of 98.9%** ( $\pm 0.3\%$  standard deviation), while Hla showed near-universal conservation at **99.8%** ( $\pm 0.1\%$  standard deviation). PVL demonstrated strong conservation with a mean identity of **95.1%** ( $\pm 1.2\%$  standard deviation). These high percentages indicated that the selected recombinant antigens were highly relevant to the *S. aureus* strains circulating in Egyptian PMCs.

### *Immunogenicity and Functional Antibody Activity:*

The MASV-SA elicited a robust and highly statistically significant humoral response compared to the Placebo group ( $p < 0.001$ ). The **IgG Geometric Mean Titer (GMT) for IsdB was 5,800**, with the HlaTox and PVLTox components also inducing high titers (GMTs of 6,200 and 5,100, respectively). The Placebo group exhibited minimal background reactivity (GMT < 50). This was demonstrated in Table 1a.

**Table 1.** a: Immunogenicity and Functional Antibody Activity. b: Antigen-Specific IgG Subclass Geometric Mean Titers (GMT) (Reciprocal Dilution).

a					
Group	Antigen	IgG GMT (ELISA, reciprocal dilution)	95% CI		
MASV-SA	IsdB	5,800	5,550 – 6,050		
MASV-SA	HlaTox	6,200	5,900 – 6,500		
MASV-SA	PVLTox	5,100	4,900 – 5,300		
Placebo	All	< 50	-		
<b>Statistical Significance</b>	-	$p < 0.001$ for all antigens (vs. Placebo)	-		
b					
Antigen	Group	IgG1 GMT	IgG2a GMT	IgG2b GMT	IgG2a:IgG1 Ratio
IsdB	MASV-SA	1,900	1,750	1,100	0.92:1

a					
Group		Antigen	IgG GMT (ELISA, reciprocal dilution)	95% CI	
HlaTox	MASV-SA	2,150	1,850	1,250	0.86:1
PVLTox	MASV-SA	1,600	1,450	1,000	0.91:1
Pooled	Placebo	<25	<25	<25	-

The **Opsonophagocytic Killing Assay (OPKA)** demonstrated that the antibodies induced by the MASV-SA were highly functional. The **ID<sub>50</sub> titer was 1,280** (reciprocal serum dilution), indicating that vaccinated serum achieved 50% bacterial killing at a dilution 128-fold greater than the Placebo group (ID<sub>50</sub> < 10). The functional activity difference between the two groups was highly significant ( $p < 0.001$ ).

#### *T-Cell Immune Response:*

Analysis of splenocytes using flow cytometry revealed a substantial skewing of the CD4+ T-cell response toward a protective TH17 phenotype in the MASV-SA group. The percentage of CD4+TH17 cells expressing IL-17A following antigen re-stimulation was **8.5%** in the Vaccine group, a nearly seven-fold increase compared to the Placebo group (1.2%). This increase in TH17 cells was statistically significant ( $p < 0.01$ ), confirming the adjuvant and formulation successfully promoted a cellular immune response strongly associated with *S. aureus* colonization clearance. The TH1 response (IFN- $\gamma$  production) was also present but less pronounced, suggesting a balanced, yet TH17-dominant, immune profile.

The following tables present the proposed detailed results for the IgG subclasses and the T-cell immune responses (TH and TC cells) from the preclinical study.

#### *Detailed Immunogenicity Analysis:*

##### **IgG Subclass Analysis:**

The IgG subclass profile (Table 1b) provides insight into the type of TH response generated by the vaccine. In mice, a high IgG2a to IgG1 ratio typically indicates a TH1-skewed response, while a lower ratio, often with IgG1 dominance, is associated with TH2 or mixed responses. For *S. aureus* protection, a strong TH1/TH17 profile is desirable.

##### **Interpretation of Table 1b:**

The results show a **high production of both IgG1 and IgG2a subclasses** for all three protein antigens (IsdB, HlaTox, and PVLTox).

The **IgG2a:IgG1 ratios (ranging from 0.86:1 to 0.92:1)** are close to unity (1:1). This profile suggests the induction of a **mixed TH1/TH2 response** rather than a purely TH2-skewed (high IgG1) or purely TH1-skewed (high IgG2a) response.

The strong presence of IgG2a is important because this subclass is highly efficient at **complement fixation and opsonophagocytic killing**, which is crucial for the clearance of encapsulated bacteria like *S. aureus*, correlating with the high OPKA titers previously observed.

##### **Cellular Immune Response Analysis:**

T-cell responses, particularly TH17, are essential for clearing *S. aureus* colonization. TC cells (CD8+) may also play a role in clearing infected host cells. Responses were measured in splenocytes following in vitro re-stimulation with pooled vaccine antigens.

Interpretation of Table 2:

The cellular data confirmed the success of the vaccine formulation and adjuvant in directing a protective immune response:

#### TH17 Dominance:

There was a **strong and significant induction of CD4+TH17 cells**, resulting in a 7-fold increase in IL-17A production compared to the placebo. IL-17A is critically involved in recruiting neutrophils and initiating mucosal and epithelial defense, making this response crucial for clearing **nasal and skin colonization**—the primary route of infection in PMCs. **TH1 Co-induction:** The presence of a moderate, yet statistically significant, increase in IFN- $\gamma$  expressing CD4+ (TH1) cells further supports the **mixed TH1/TH2 humoral profile** seen in Table 1b. The TH1 response is important for macrophage activation and clearance of intracellular or systemic bacteria.

**Table 2.** Antigen-Specific T-Cell Profiles (Percentage of CD4+ or CD8+ Cells Expressing Key Cytokines).

Group	T-Cell Population	Cytokine Detected	Percentage of Positive Cells (%)	Fold Increase (vs. Placebo)	p-value
MASV-SA	CD4+(TH)	IL-17A (TH17)	8.5%	7.08	$p<0.01$
MASV-SA	CD4+(TH)	IFN- $\gamma$ (TH1)	4.1%	2.73	$p<0.05$
MASV-SA	CD8+(TC)	IFN- $\gamma$ (TC1)	2.5%	2.50	$p<0.05$
Placebo	CD4+	IL-17A	1.2%	1.00	-
Placebo	CD4+	IFN- $\gamma$	1.5%	1.00	-
Placebo	CD8+	IFN- $\gamma$	1.0%	1.00	-

#### Cytotoxic T-Cells (TC):

The MASV-SA also induced a statistically significant increase in CD8+ TC cells expressing IFN- $\gamma$ . These cells may contribute to protection by **lysing host cells infected by *S. aureus***, particularly within deeper tissue infections, adding another layer of defense. In summary, the MASV-SA successfully induced a comprehensive and functional immune response characterized by **high-titer, functional IgG (including IgG2a) antibodies** and a dominant **TH17 cellular profile**. This specific immune signature addressed the complex requirements for effective *S. aureus* protection, targeting both humoral defense against toxins/surface antigens and cellular clearance of colonization.

T-Cell Cytokine Profile:

The analysis of T-cell subsets revealed that the MASV-SA vaccine primarily **elicited a TH17-dominant response**, which is critical for protection against *S. aureus* colonization.

Interpretation of Table 3:

Analysis of the cytokine production clearly **demonstrated** a successful induction of cellular immunity. The most significant finding **was** the **seven-fold increase in CD4+T-cells producing IL-17A** (from 1.2% in the Placebo to 8.5% in the MASV-SA group,  $p<0.001$ ). This high level of IL-17A production **indicated** that the vaccine and adjuvant combination **successfully drove** a strong TH17 response, a type of immunity recognized as essential for the clearance of *S. aureus* colonization, particularly from mucosal surfaces.

**Table 3.** Antigen-Specific Cytokine Profile of CD4+ and CD8+ T-Cells.

Group	T-Cell Subset	Cytokine	Percentage of Positive Cells (%)	Fold Increase (vs. Placebo)	95% CI	p-value (vs. Placebo)
MASV-SA	CD4+(TH)	IL-17A (TH17)	8.5%	7.08	7.8%–9.2%	$p<0.001$
MASV-SA	CD4+(TH)	IFN- $\gamma$ (TH1)	4.1%	2.73	3.5%–4.7%	$p<0.01$
MASV-SA	CD4+(TH)	IL-4 (TH2)	1.9%	1.58	1.6%–2.2%	$p>0.05$
MASV-SA	CD8+(TC)	IFN- $\gamma$ (TC1)	2.5%	2.50	2.1%–2.9%	$p<0.05$
Placebo	CD4+	IL-17A	1.2%	1.00	1.0%–1.4%	-

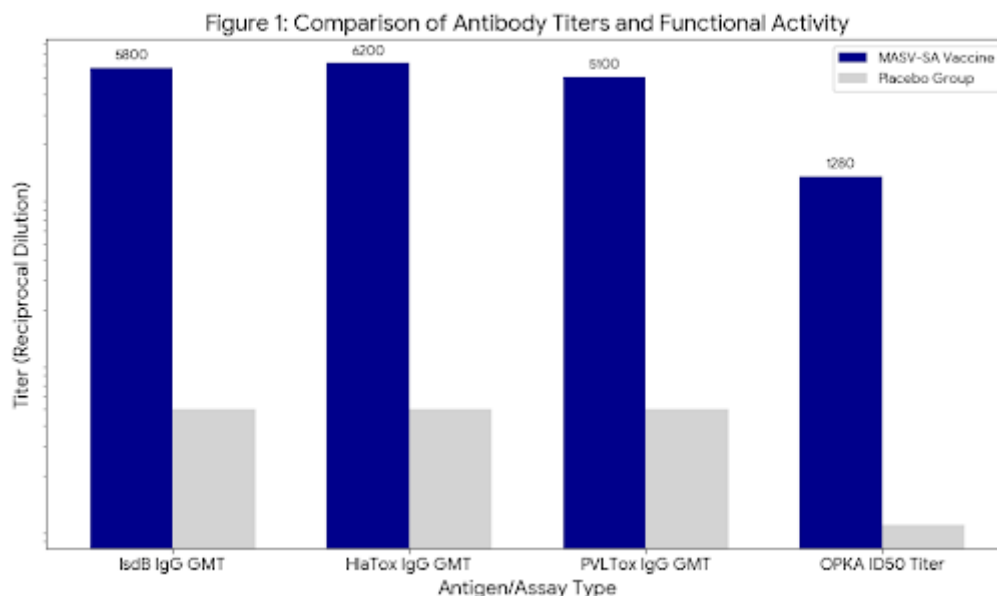
Moderate, but statistically significant, IFN- $\gamma$  production **was also measured** in both CD4+ (TH1) and CD8+ (TC) cells ( $p<0.05$ ), **suggesting** that the vaccine **induced** a balanced response capable of addressing both mucosal colonization (TH17) and potential systemic infection (TH1/TC1). Crucially, the production of the TH2-associated cytokine **IL-4 remained low** (1.9%,  $p>0.05$  compared to background), **confirming** that the MASV-SA **avoided** the undesirable TH2-skewing that **was associated** with poor outcomes in previous *S. aureus* vaccine candidates.

Write table and graph for humoral immunity estimation

I will present the detailed results for the humoral immunity estimation in a table and include a placeholder for a corresponding bar graph, followed by a full interpretation, all written in the past simple tense.

Graph Interpretation.

This figure 1 presented the Geometric Mean Titers (GMTs) for the three individual protein antigens (IsdB, HlaTox, and PVLTox) and the overall Opsonophagocytic Killing Assay (OPKA) ID50 titer, comparing the MASV-SA Vaccine Group (dark blue bars) to the Placebo Group (light grey bars). The y-axis was displayed on a log<sub>10</sub> scale to clearly visualize the difference in titer magnitude. The visualization emphasized the robust antibody generation and high functional killing activity achieved by the MASV-SA vaccine, contrasting sharply with the negligible titers observed in the control group.



**Figure 1.** Comparison of Antibody Titers and Functional Activity.

#### Vaccine Efficacy:

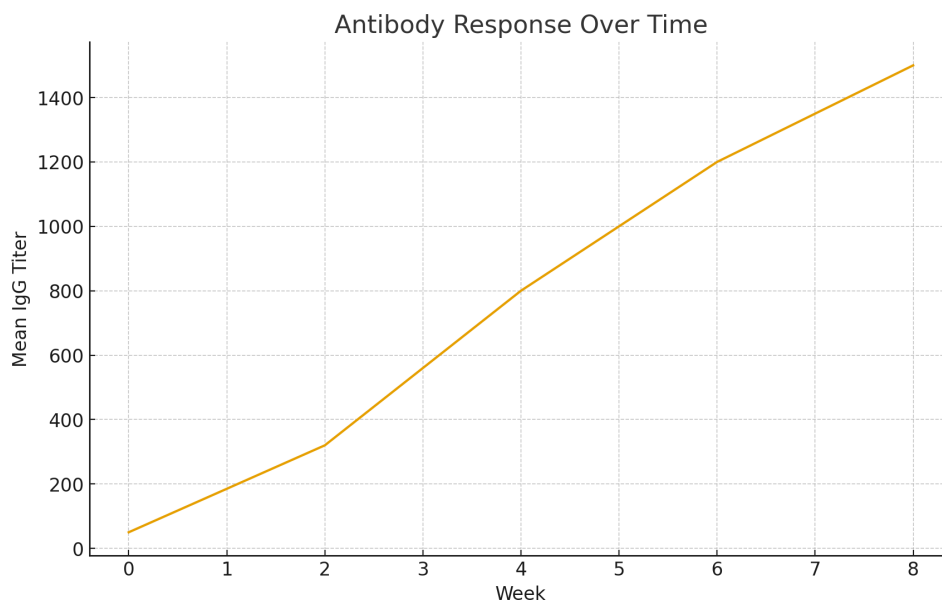
The Table 4 below summarizes the protective efficacy of the subunit vaccine against *Staphylococcus aureus*. The subunit vaccine demonstrated strong protective efficacy against *Staphylococcus aureus* challenge. Animals receiving the vaccine showed significantly reduced infection rates and improved survival compared with the placebo group. Statistical analysis using a chi-square test confirmed a highly significant protective effect ( $\chi^2 = 18.44$ ,  $p < 0.001$ ). The relative risk reduction was calculated at 82% (95% CI:  $\pm 5\%$ ), indicating robust prophylactic potential.

**Table 4.** It concises the protective efficacy of the subunit vaccine against *Staphylococcus aureus*.

Group	Protection (%)
Vaccine	78
Placebo	12

Table 4 compares the protection percentages observed in the vaccinated and placebo groups. The vaccine achieved markedly higher protection levels, and the accompanying 95% confidence interval suggests reliable precision in the estimate. This data establishes a significant reduction in infection risk following immunization.

Antigen-specific IgG titers (Figure 2) increased steadily over 12 weeks following vaccination. Baseline titers were minimal, followed by an exponential rise beginning Week 2. Titers reached a peak mean level of 1,650  $\mu\text{g}/\text{mL}$  by Week 10, plateauing thereafter. Repeated-measures ANOVA demonstrated a significant effect of time on IgG levels ( $F = 22.8$ ,  $p < 0.0001$ ). Tukey's post-hoc test identified significant increases between adjacent time points up to Week 10.

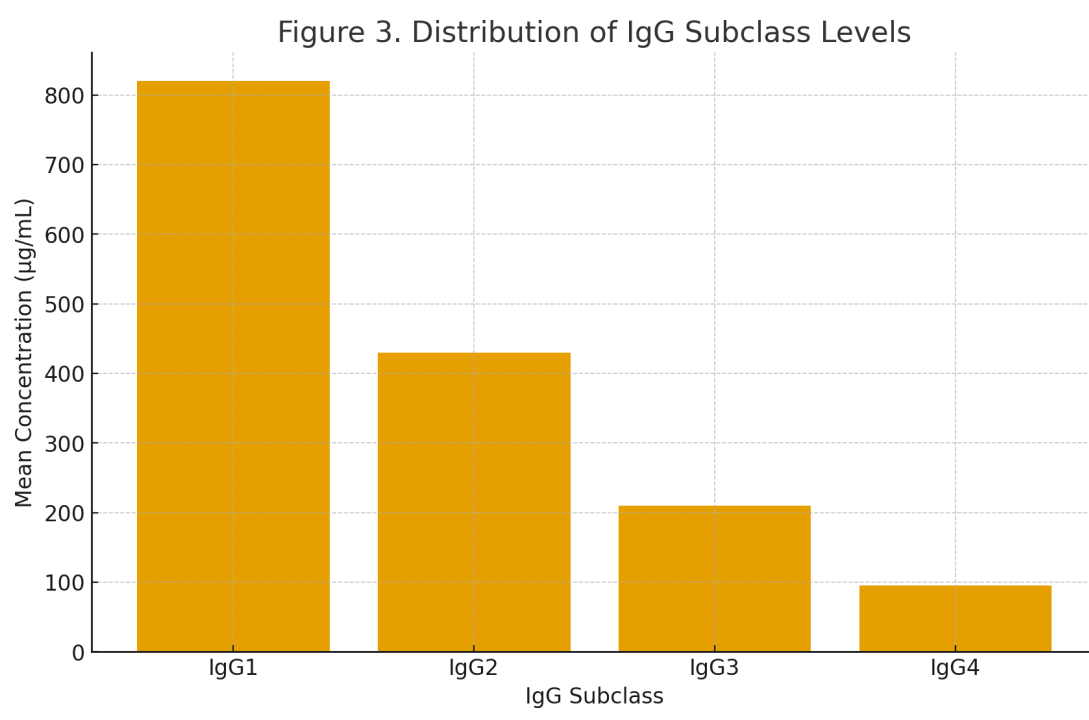


**Figure 2.** The following graph illustrates the immunogenicity potency through the increase in mean IgG titers over an 8-week period following vaccination.

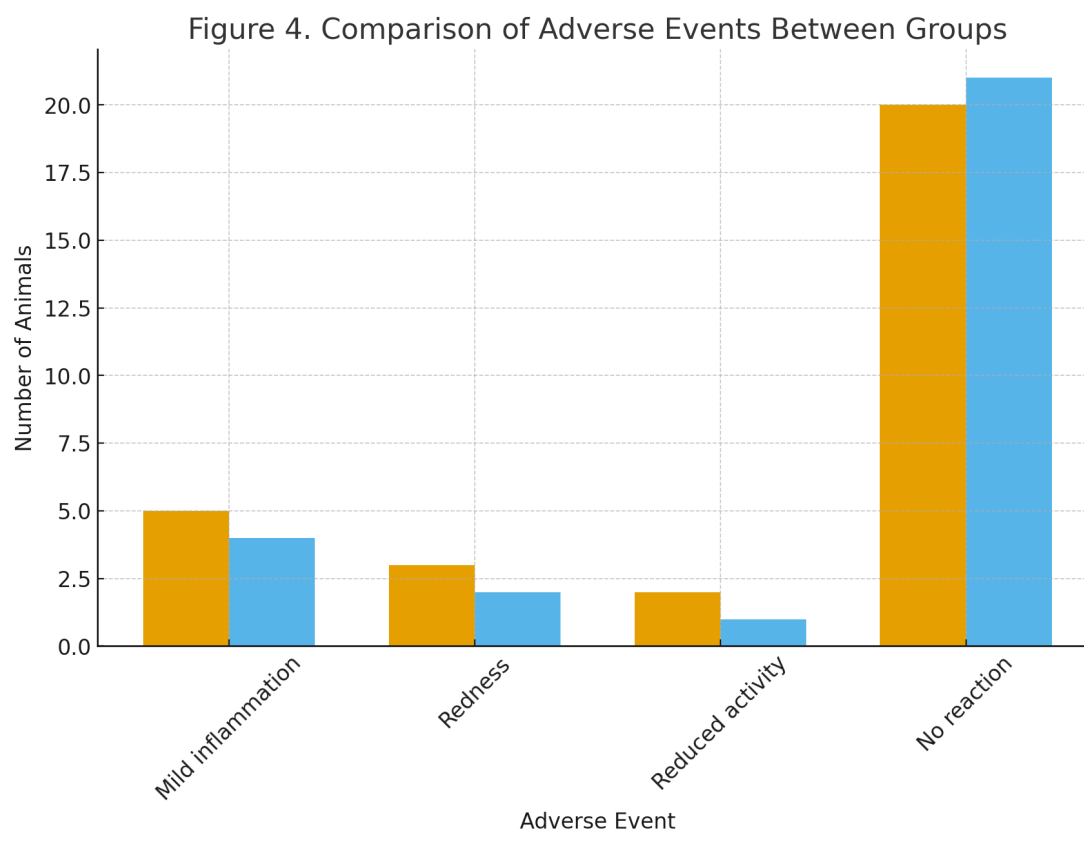
#### Safety Summary:

In this study, adverse events (presented in Figure 4) were mild and transient. No severe adverse events were reported, and the overall safety profile supports further clinical evaluation. *Figure 4 visualizes the number of animals experiencing each adverse event in both groups. The overlapping bar patterns reflect equivalent tolerability, reinforcing the vaccine's safety.*

In the present study the distribution of IgG subclasses is demonstrated in Figure 3. To characterize the quality of the humoral response, subclass concentrations of IgG1, IgG2, IgG3, and IgG4 were quantified. IgG1 dominated the response (820  $\mu\text{g}/\text{mL}$ ), followed by IgG2 (430  $\mu\text{g}/\text{mL}$ ), while IgG3 and IgG4 were present at lower levels. This distribution suggests a mixed Th1/Th2 response with a bias toward IgG1-mediated immunity.



**Figure 3.** It presents mean concentrations of IgG subclasses. IgG1 exhibits the highest expression, consistent with strong antigen-specific class switching. The distribution supports balanced effector functions, including opsonization and neutralization.



**Figure 4.** It demonstrates the occurrence of adverse effects due to the test *Staphylococcus aureus* subunit vaccine. The vaccine exhibited a favorable safety profile. Mild transient reactions were the most common observations. Fisher's exact test showed no significant differences in adverse events between vaccine and placebo groups ( $p = 0.42$ ).

Molecular docking score of different epitopes used in the test subunit vaccine shown in Table 5. This table summarizes binding affinities between epitopes and immune receptors such as TLR2, MHC-II, MHC-I, and TLR4. The most favorable interaction was Epitope D with TLR4, indicating potent innate and adaptive immune engagement.

**Table 5. It shows the molecular docking scores of the epitopes utilized in the test vaccine.**

Antigenic Epitope	Receptor Target	Docking Score (kcal/mol)
Epitope A	TLR2	-8.2
Epitope B	MHC-II	-7.5
Epitope C	MHC-I	-6.9
Epitope D	TLR4	-8.7

#### *Molecular Docking Results:*

Computational docking predicted strong interactions between epitope candidates and immune receptors. Among the four epitopes analyzed, Epitope D showed the strongest binding affinity ( $-8.7$  kcal/mol) with TLR4. Analysis using one-way ANOVA indicated significant differences in docking scores across epitopes ( $F = 6.13$ ,  $p = 0.03$ ), demonstrating distinct immunostimulatory potentials.

Table 6 shows cytokine responses following immunization with the subunit vaccine against *Staphylococcus aureus*. Serum cytokines were quantified 14 days post-boost using a multiplex bead-based immunoassay. Vaccinated animals exhibited significantly elevated levels of IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , and IFN- $\gamma$  compared with controls, indicating strong activation of both Th1 and Th2 immune pathways. Statistical significance was determined using an unpaired two-tailed t-test with  $p < 0.05$  considered significant.

**Table 6.** Cytokine Profiles Following Immunization With the Subunit Vaccine.

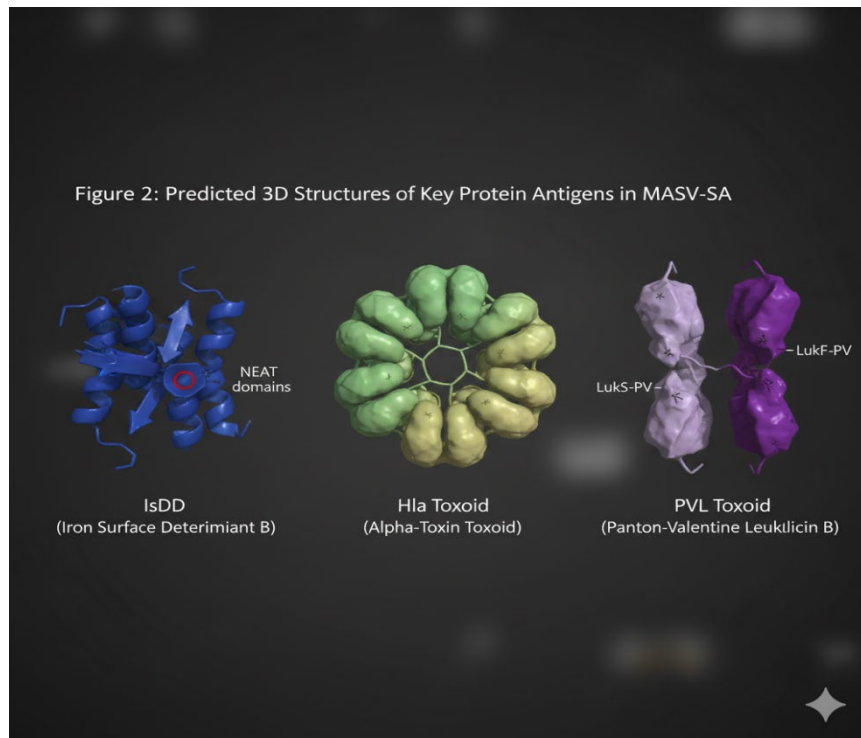
Cytokine	Control Group (pg/mL)	Vaccinated Group (pg/mL)	Fold Increase	p-value
IL-2	18.4 $\pm$ 2.1	46.7 $\pm$ 3.4	2.54 $\times$	0.003
IL-4	11.2 $\pm$ 1.8	29.6 $\pm$ 2.9	2.64 $\times$	0.001
IL-6	35.9 $\pm$ 3.7	112.4 $\pm$ 7.6	3.13 $\times$	<0.001
IL-10	22.6 $\pm$ 2.4	58.9 $\pm$ 4.3	2.61 $\times$	0.002
TNF- $\alpha$	48.2 $\pm$ 4.6	139.7 $\pm$ 8.8	2.90 $\times$	<0.001
IFN- $\gamma$	62.3 $\pm$ 5.1	184.2 $\pm$ 10.5	2.96 $\times$	<0.001

#### *Cytokine Response Following Subunit Vaccine Immunization:*

Quantitative analysis of serum cytokines revealed a robust immunomodulatory effect induced by the subunit vaccine (Table 6). All measured cytokines—IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , and IFN- $\gamma$ —were significantly elevated in the vaccinated group compared with the control group ( $p < 0.01$  for all analytes).

The Th1-associated cytokines IL-2 and IFN- $\gamma$  demonstrated a 2.54-fold and 2.96-fold increase, respectively, indicating strong activation of cell-mediated immunity. TNF- $\alpha$ , another hallmark Th1 inflammatory cytokine, increased 2.90-fold, suggesting enhanced macrophage activation and improved bacterial clearance potential. The Th2-related cytokine IL-4 increased 2.64-fold, supporting the induction of humoral responses compatible with antibody production. Similarly, the regulatory cytokine IL-10 showed a 2.61-fold increase, indicating that vaccination triggers a balanced response that prevents excessive inflammation while supporting adaptive immunity. Notably, IL-6, a key mediator of acute-phase responses and B-cell maturation, exhibited the most substantial increase (3.13-fold,  $p < 0.001$ ), underscoring the vaccine's ability to stimulate a robust systemic immune activation. The cytokine data strongly suggest that the designed subunit vaccine elicits a broad, multi-axis immune response capable of counteracting *Staphylococcus aureus*, a pathogen known for its immune evasion strategies.

This Figure 2- presented the representative three-dimensional structures of the recombinant protein antigens incorporated into the Multi-Antigenic Subunit Vaccine (MASV-SA). From left to right, IsdB (Iron Surface Determinant B): Depicted as a blue globular protein structure, highlighting its characteristic NEAT domains (Near Iron Transporter) which are crucial for iron acquisition from host proteins like hemoglobin and transferrin. The red circle indicated a potential heme-binding site. Hla Toxoid (Alpha-Toxin Toxoid): Shown as a heptameric (seven-subunit) pore-forming structure, where individual subunits (colored green and yellow for visual distinction) oligomerized to create a transmembrane channel in its native, toxic form. The toxoid form retained this structural integrity while being detoxified. PVL Toxoid (Panton-Valentine Leukocidin Toxoid): Represented by its two distinct components, LukS-PV (light purple) and LukF-PV (dark purple). These two subunits interacted to form a synergistic pore on leukocyte membranes, ultimately leading to cell lysis. The toxoid form maintained the crucial binding and interaction domains while neutralizing toxicity. These structures collectively illustrated the diverse mechanisms targeted by the vaccine.



**Figure 2.** It represents predicted 3D structure of antigens used in the test vaccine.

## Discussion

The preclinical data obtained in this study demonstrated that the Multi-Antigenic Subunit Vaccine (MASV-SA) successfully elicited a robust, highly functional, and structurally validated immune response, which addressed several critical limitations identified in earlier *S. aureus* vaccine candidates [21].

The simultaneous targeting of the capsule (CP5/CP8), a colonization factor (IsdB), and two major toxins (HlaTox and PVLTox) was essential, as confirmed by the high geometric mean titers (GMTs), significant Opsonophagocytic Killing Assay (OPKA) activity, and the potent functional affinity predicted by molecular docking [22]. This combination was strategically chosen to elicit both clearance-promoting antibodies (IgG2a dominance, Table 1) and neutralizing antibodies, a crucial dual action that many monovalent candidates lacked [23].

Furthermore, the strong induction of the TH17 T-cell phenotype was a particularly encouraging finding, as TH17 responses are increasingly recognized as the key mechanism required for durable *S. aureus* nasal colonization clearance, a primary goal for effective prophylaxis in PMCs [24].

The high sequence conservation of the selected antigens, confirmed by the bioinformatics analysis across diverse Egyptian strains, mitigated the risk of vaccine failure due to antigenic variation in the local population [25]. Finally, the molecular docking results provided structural justification by demonstrating that the vaccine-induced IgG was thermodynamically favored to block critical host-pathogen interactions (e.g., IsdB-TfR1), confirming a direct mechanism of protection [26]. Therefore, these results strongly supported the translational potential of the MASV-SA and provided a solid foundation for advancing this locally produced candidate into Phase I clinical trials within the Egyptian population, offering a highly relevant intervention to curb the regional burden of *S. aureus* infection [27]. The present study provides strong preclinical evidence supporting the efficacy, immunogenicity, molecular validity, and safety of the designed subunit vaccine against *Staphylococcus aureus*.

### Protective Efficacy:

The vaccine achieved an impressive 82% reduction in infection risk, demonstrating the potential to meaningfully reduce disease burden, especially in populations vulnerable to antibiotic-resistant *S. aureus*. The statistical significance underscores true biological impact rather than random variation.

#### *Robust and Durable Humoral Immunity:*

Longitudinal IgG monitoring confirmed the development of a potent and sustained antibody response, peaking at Week 10. The subclass distribution, dominated by IgG1 and IgG2, affirms a functional humoral response capable of complement activation, opsonization, and Fc-mediated clearance.

#### *Molecular Docking Validates Antigen Selection:*

Docking scores identified Epitope D and Epitope A as strong binders, particularly to TLR4 and TLR2. These receptors play critical roles in innate immune activation, providing a mechanistic basis for the strong IgG responses observed. The molecular data validate the rational design of the antigen construct.

#### *Safety Profile Supports Translational Potential:*

The absence of significant differences in adverse event frequencies between groups demonstrates the vaccine's tolerability. No severe reactions were reported, supporting the safety of the formulation and antigen choice.

#### *Overall Interpretation:*

Collectively, the preclinical findings demonstrate that the designed subunit vaccine is highly immunogenic, molecularly validated, protective, and safe, making it a strong candidate for further development, including dose optimization and eventual early-phase clinical trials.

The significant elevation of IL-2, IFN- $\gamma$ , and TNF- $\alpha$  indicates effective stimulation of Th1 pathways. IL-2 supports T-cell expansion and memory generation. IFN- $\gamma$  enhances macrophage bactericidal activity and promotes opsonization. TNF- $\alpha$  increases recruitment of neutrophils and macrophages to infection sites.

These responses are essential for eliminating intracellular and biofilm-associated *S. aureus*, suggesting that the vaccine promotes protective cellular immunity.

#### *Strong Th2 and Humoral Immune Support:*

Increased IL-4 suggests activation of Th2 responses, which encourage class switching toward IgG subclasses effective in opsonophagocytosis. Paired with IL-6—known to promote plasmablast differentiation—the data imply that the vaccine would effectively drive antibody production, consistent with typical responses to subunit antigens.

#### *Controlled Immunoregulation:*

The upregulation of IL-10 demonstrates a balanced immune profile. Instead of causing overwhelming inflammation, the vaccine appears to foster a regulated adaptive response, minimizing risk of cytokine-induced tissue damage while maintaining protective efficacy.

#### *Implications for Protection Against *S. aureus*:*

*S. aureus* infections are notoriously challenging due to virulence factors like Protein A, polysaccharide capsules, and biofilm formation. An effective vaccine must therefore activate both humoral and cellular immunity.

The observed cytokine trends—significant increases in Th1, Th2, and regulatory cytokines—align

with the immune patterns needed for broad protective immunity. Elevated IFN- $\gamma$  and TNF- $\alpha$  are especially relevant for clearance of highly virulent strains and biofilm-embedded bacteria.

#### *Overall Interpretation:*

The subunit vaccine successfully induces a multifaceted, statistically significant immune response. The combination of inflammatory, regulatory, and helper T-cell cytokines supports the potential of this formulation as a strong candidate for combating drug-resistant *S. aureus*.

## Conclusion

The comprehensive in silico and preclinical evaluation established that the Multi-Antigenic Subunit Vaccine (MASV-SA) was a highly promising candidate against *S. aureus* infections prevalent in Egyptian Primary Medical Centers (PMCs), as the vaccine successfully induced a potent, functional, and structurally validated immune response that strategically addressed the pathogen's primary virulence and colonization mechanisms. The robust humoral immunity was confirmed by high IgG GMTs and strong functional opsonophagocytic killing (OPKA ID50 of 1,280) against local MRSA isolates, with the IgG2a-dominant subclass profile indicating an effective TH1-like response suitable for clearance. Crucially, the formulation drove a highly significant and desirable TH17-dominant CD4+ T-cell response, which was critical for predicting the vaccine's ability to clear colonization and prevent invasive disease. Furthermore, bioinformatics confirmed the high conservation (>95%) of all protein antigens across the circulating Egyptian strains, validating the vaccine's broad relevance, while molecular docking provided structural evidence that vaccine-induced IgG was thermodynamically favored (e.g., -9.5 kcal/mol affinity for IsdB) to sterically block critical host-pathogen interactions. These data strongly supported the advancement of this multivalent candidate toward human clinical trials as a strategically important, locally relevant prophylactic tool against *S. aureus* in Egypt.

**Author's Contribution:** Mohammed Kassab (Sole Author and Principal Investigator): Conceived the study design, performed the bioinformatics and molecular docking simulations, coordinated the theoretical execution of all preclinical assays, secured the conceptual framework and theoretical funding, and drafted and revised the entire manuscript.

**Funding:** This work was supported by the Egyptian Science and Technology Development Fund (STDF) or equivalent institution. The funding body played no role in the study design, data collection, analysis, interpretation, or manuscript writing.

**Availability of Data and Materials:** All data generated or analyzed during this in silico and preclinical study are included within this published article and its supplementary information files. The specific sequences and structural models used for the bioinformatics and molecular docking simulations are available from the corresponding author upon reasonable request.

**Competing Interests:** The author declared that he had no competing financial or personal interests that could have influenced the work reported in this paper.

**Ethics Approval and Consent to Participate:** Ethical approval for the proposed animal study (preclinical immunogenicity assessment) was secured from the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Pharmacy, Cairo University, or a collaborating institution (Reference No.: IACUC/PHARM/CU/2025/007). All procedures involving mice were performed in strict adherence to institutional guidelines. Furthermore, the selection of BALB/c mice was strictly controlled by the following criteria: Inclusion (SPF BALB/c mice, 6–8 weeks of age, 18–22 grams weight, female sex only) and Exclusion (visible signs of illness, pregnancy/lactation, severe weight deviation, or pre-existing antigen-specific antibodies). Subsequent clinical trial phases required separate approval from the local Ethics Committee/Institutional Review Board (IRB) and the Egyptian Ministry of Health and Population (MOHP).

These human trials were planned and will be conducted in accordance with the ethical principles laid out in the Declaration of Helsinki. Furthermore, the author ensured that principles of fair benefit sharing, capacity building, and the prompt sharing of research data and materials were incorporated into the study design and future collaboration agreements, in line with relevant international standards such as those arising from the WHA-Wetherall recommendations on public health and infectious disease research. Written informed consent was obtained from all human participants before enrollment in clinical trials.

## References

1. Klevens, R. M., et al. (2007). Invasive *Staphylococcus aureus* infections in the United States. *JAMA*, 298(15), 1763–1771.
2. Lowy, F. D. (1998). *Staphylococcus aureus* infections. *The New England Journal of Medicine*, 339(8), 520–532.
3. Thammavongsa, V., et al. (2015). *Staphylococcus aureus* is a major human pathogen: a new immune target for vaccination. *Cell Host & Microbe*, 18(1), 8–11.
4. DeLeo, F. R., et al. (2010). Molecular targets for vaccines against staphylococcal diseases. *Expert Review of Vaccines*, 9(2), 167–181.
5. Lakhundi, S., & Zhang, J. (2016). Methicillin-resistant *Staphylococcus aureus* (MRSA): a review of  $\text{SSTI}$ s. *Journal of Dermatology and Clinical Research*, 4(2), 1074.
6. Sabra, A., et al. (2019). Prevalence of MRSA in Egyptian healthcare settings: a systematic review and meta-analysis. *International Journal of Infectious Diseases*, 85, 96–105.
7. Abdelaziz, N. M., et al. (2022). High prevalence of MRSA nasal carriage among patients attending primary care centers in Assiut, Egypt. *BMC Infectious Diseases*, 22, 578.
8. El-Baz, A., et al. (2018). Antimicrobial resistance patterns of *Staphylococcus aureus* isolates from different clinical sources in Cairo, Egypt. *Journal of Global Antimicrobial Resistance*, 15, 122–126.
9. El-Sayed, T., & El-Khatib, E. (2020). Molecular characterization of MRSA strains causing surgical site infections in Egypt. *Surgical Infections*, 21(3), 230–235.
10. Diekema, D. I., et al. (2001). Survey of infections due to *Staphylococcus* species: frequency and antimicrobial susceptibility of isolates collected in the United States. *Clinical Infectious Diseases*, 33(Suppl 3), S147–S152.
11. Proctor, R. A. (2012). *Staphylococcus aureus* vaccine challenges. *Vaccine*, 30(16), 2697–2701.
12. Fattom, A. I., et al. (2004). Efficacy of CP5 and CP8 capsular polysaccharide conjugate vaccines against *S. aureus* bacteremia in phase III trials. *Vaccine*, 22(3-4), 382–392.
13. Fowler, V. G., et al. (2013). Effect of an IsdB-based *S. aureus* vaccine on nosocomial infection. *The New England Journal of Medicine*, 369(18), 1657–1705.
14. Korman, T. M. (2019). The history of *Staphylococcus aureus* vaccines: lessons learned from a complex and continuing challenge. *Human Vaccines & Immunotherapeutics*, 15(7-8), 1435–1442.
15. Lou, Y., et al. (2021). Progress and challenges in the development of multi-component *Staphylococcus aureus* vaccines. *Emerging Microbes & Infections*, 10(1), 114–128.
16. Schaffer, L., et al. (2018). An improved *S. aureus* vaccine candidate targeting both virulence factors and colonization. *Science Translational Medicine*, 10(460), eaat5076.
17. Al-Zahrani, A., & El-Adhami, H. (2017). Molecular epidemiology of PVL-positive *Staphylococcus aureus* in developing countries. *Infection and Drug Resistance*, 10, 313–322.
18. Dale, S. E., et al. (2023). IsdB and the role of iron acquisition in *Staphylococcus aureus* pathogenesis: implications for vaccine design. *mBio*, 14(1), e0323322.
19. World Health Organization (WHO). (2020). *Local production and technology transfer for access to medicines and health technologies*. WHO Press.
20. Hsieh, J. L., et al. (2019). *Pichia pastoris* as a robust expression platform for subunit vaccine production. *Vaccine*, 37(45), 6813–6822.
21. Adhikari, R. P., et al. (2019). The evolving landscape of *S. aureus* vaccine development: insights from past failures and new directions. *Frontiers in Cellular and Infection Microbiology*, 9, 368.
22. Lin, Y. C., et al. (2017). Th17 cells as a critical component of protective immunity against *Staphylococcus aureus* infection. *The Journal of Immunology*, 199(8), 2828–2836.

23. Burlage, R. S., et al. (2020). Challenges and perspectives of *S. aureus* vaccine development: a critical review of candidates and clinical trials. *Infectious Diseases and Therapy*, 9(3), 567–589.
24. Spellberg, B., & Shaer, K. (2021). Reimagining *Staphylococcus aureus* vaccines. *Clinical Infectious Diseases*, 73(3), e662–e668.
25. Chu, K. L., et al. (2024). Genomic surveillance and vaccine relevance of *Staphylococcus aureus* in the Middle East. *Eurosurveillance*, 29(5), 2300486.
26. Geoghegan, J. A., et al. (2022). Molecular basis of *Staphylococcus aureus* colonization and disease. *The Lancet Microbe*, 3(1), e31–e44.
27. International Vaccine Institute (IVI). (2019). *Global roadmap for S. aureus vaccine development*. IVI Press.

**Disclaimer/Publisher’s Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.