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In silico Evaluation, Cloning, and Expression of Omp22 as a Promising Vaccine Candidate against *Acinetobacter baumannii*

Samira Sabzi[1], Farzad Badmasti[2], Narjes Noori Goodarzi[3], Mehri Habibi[1], Shahla Shahbazi[1], Mohammad Reza Asadi Karam[1]*, Mehdi Farokhi[4]*

[1]Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran. [2]Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran. [3]Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. [4]National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran.

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*** Corresponding Authors:**
Mohammad Reza Asadi Karam, Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran
Email: m_asadi12@yahoo.com
Tel/Fax: +982164112221

Mehdi Farokhi,
National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran, Iran
Email: m_farokhi@pasteur.ac.ir
Tel/Fax: +982164112358

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ABSTRACT

Introduction: There is currently no approved vaccine available for *Acinetobacter baumannii*, an important agent of nosocomial infections. Recently, Omp22 from *A. baumannii* has been identified as a vaccine candidate that stimulates effective immune responses in mice. However, limited data is available about this protein. This study aimed to comprehensively analyze the immunoinformatic properties of Omp22 and its expression *in vitro*. **Methods:** The protein sequence of Omp22 was scanned for subcellular localization, antigenicity, allergenicity, homology to human proteome, physiochemical characteristics, linear and conformational B-cell epitopes, MHC binding sites, tertiary structure prediction, and molecular dockings. Additionally, the gene encoding omp22 was cloned into the pET-28a (+) vector and the expression level was optimized. **Results:** Omp22 (22.48 kDa, pI of 9.30) belongs to the outer membrane proteins superfamily without transmembrane helices. Omp22 was predicted to be a non-allergenic antigen with appropriate stability. Two linear B-cell epitopes were identified, as well as 108 MHC-I and 50 MHC-II binding sites. Three conformational B-cell epitopes were identified through 3D structure prediction, and molecular docking analysis showed desirable interactions in the docked complexes. The optimized expression of the recombinant Omp22 was successfully achieved. **Conclusion:** This study represents a significant step towards developing an Omp22-based vaccine candidate against *A. baumannii*. However, further experimental analyses are still needed.

INTRODUCTION

Acinetobacter baumannii is a Gram-negative bacterium that commonly causes healthcare-associated infections, including pneumonia, meningitis, bloodstream infections, soft tissue infections, and urinary tract infections [1]. It is classified as an ESKAPE pathogen, which the World Health Organization (WHO) has reported as a critical priority pathogen [2]. The management of *A. baumannii* infections is challenging due to the rise of drug-resistant strains, known as multidrug-resistant (MDR) strains [3, 4]. Despite the prevalence of MDR and extensively drug-resistant (XDR) infections, effective antibiotics for treating these strains have not been developed by the pharmaceutical industry. Consequently, there is a growing concern that nosocomial infections caused by *A. baumannii* will soon become untreatable [5].

Vaccination could provide an affordable solution to reduce the clinical and financial burden of these infections [6, 7]. Among different types of vaccines, subunit vaccines are considered safe and effective due to their ability to stimulate specific immune responses [8]. Outer membrane proteins (OMPs) are particularly promising vaccine targets due to their crucial roles in bacterial survival, virulence, cell adhesion, and host invasion [9]. Several protein-based antigens such as OmpA [10], OmpW [11], BamA [12], Bap [13], Ata [14], OprF [15], Omp34 [16], and phospholipase D [17] have been evaluated as potential vaccine candidates. Despite these efforts, no approved vaccine against *A. baumannii* currently exists. More recently, Omp22 has emerged as a highly promising candidate that elicits effective immune responses in experimental mice [18-20]. It is believed that Omp22 plays a significant role in the biogenesis of outer membrane vesicles and interacts with peptidoglycan, although the exact biological functions of Omp22 remain unknown [18].

Advancements in bioinformatics and the availability of immunoinformatic databases have facilitated the evaluation of potential immunogenic candidates through *in silico* approaches

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[21-23]. Although there have been various immunoinformatic studies on vaccine candidates against *A. baumannii*, there is limited data on the potential of Omp22 to be utilized as an effective component in the development of a protective vaccine. This study aims to comprehensively evaluate Omp22 through *in silico* analysis and optimize the expression of recombinant Omp22 *in vitro*.

MATERIALS AND METHODS

Protein Retrieval and Subcellular Localization

The protein sequence of Omp22 was extracted from both NCBI (Accession number: WP_001202415; https://www.ncbi.nlm.nih.gov/protein) and UniProt databases (Accession number: D0C9R5; https://www.uniprot.org) in FASTA format for further investigations. The protein sequence was imported to the PSORTb v.3.0.2 online server (www.psort.org/psortb/) for evaluation of subcellular localization [24]. This server used a threshold of above 7.5 to determine protein localization efficiently [25]. The TMHMM Server v. 2.0 web tool was applied (http://www.cbs.dtu.dk/services/TMHMM/) to identify transmembrane helices [26].

Antigenicity and Allergenicity Determination

The antigenic properties of Omp22 were determined by VaxiJen online server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with a threshold of ≥ 0.4. VaxiJen is the first software that predicts antigenic properties via the machine learning method [27]. Furthermore, the allergenicity of protein was assessed by the AlgPred 2.0 web tool (https://webs.iiitd.edu.in/raghava/algpred2/batch.html) with a threshold of ≥ 0.5 [28].

Similarity with the Human Proteome

To prevent auto-immune responses resulting from the sequence similarity of Omp22 with human proteome (*Homo sapiens* taxid: 9606), an online PSI-BLAST server was applied, provided by the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) (Identity > 15%, Max score > 100, E-value < 10^{-3}) [29]. Identification of sequence similarity via PSI-BLAST is more sensitive than normal BLAST when they are distantly related to the query sequence.

Physiochemical Characteristics of Omp22

The physiochemical properties including molecular weight, theoretical pI, the estimated half-life, aliphatic, and instability indexes of Omp22 were assessed by the Expasy ProtParam server (https://web.expasy.org/protparam/) [30]. The functional class of the proteins and adhesion probability were predicted through VICMpred (https://webs.iiitd.edu.in/raghava/vicmpred) and Vaxign (http://www.violinet.org/vaxign2), respectively [31].

Identification of Linear and Conformational B-cell epitopes, and MHC Binding Sites

Linear B-cell epitopes of Omp22 were predicted using BepiPred (https://services.healthtech.dtu.dk/services/BepiPred-2.0/) with a cut-off > 0.6. [32] This server was used for the prediction of B-cell epitopes through the combination of a Hidden Markov Model (HMM) and a propensity scale method. Epitopes identified by these servers were then evaluated for allergenicity and antigenicity properties. MHC-I and MHC-II binding sites were identified using the Tepitool web server, the prediction tool of the Immune Epitope Database (http://tools.iedb.org/tepitool/) [33]. For the prediction of MHC-I binding sites, a panel of 27 most frequent A&B alleles (providing the human worldwide population coverage > 95%) was used and only peptide length 9 will be included. Similarly, MHC-II binding sites were predicted with consideration of the panel of 26 most frequent alleles, and the number of overlapping residues was fixed at 10. Additionally, the ToxinPred server (https://webs.iiitd.edu.in/raghava/toxinpred/design.php) with the SVM method and default server parameters was used to determine toxic epitopes. The tertiary structure (3D) of Omp22 was predicted by the I-TASSER tool (https://zhanggroup.org/I-TASSER/) [34]. Consequently, the quality of the Omp22 3D structure was confirmed using the ProSA-Web tool (https://prosa.services.came.sbg.ac.at/prosa.php) and the Ramachandran plot [35]. ProSA-Web analysis shows possible errors in the 3D structure, while the Ramachandran plot shows the conformation psi (ψ) and phi (φ) angles of the polypeptide. In the next step, the conformational B-cell epitopes were determined using the ElliPro server (http://tools.iedb.org/ellipro/) with a threshold of ≥ 0.8 [36]. The predicted conformational B-cell epitopes were characterized on the surface of the target protein with different colors by Jmol software. Then, the conservancy of the Omp22 was determined using the ConSurf web server (https://consurf.tau.ac.il/) [37].

Molecular Dockings

Human Toll-like receptors (TLRs) are the most important membrane-bound PRRs, playing an important role in the recognition of invading pathogens by the innate immune system. *A. baumannii* interacts with host cells mostly by triggering TLR2 and TLR4 [38]. The interactions of Omp22 with TLR2 (PDB ID 6NIG) and TLR4 (PDB ID 4G8A) were evaluated using the HDOCK web server available at (http://hdock.phys.hust.edu.cn/) [39].

Cloning and Expression Optimization of Omp22

The full length of the *omp22* gene was amplified from *A. baumannii* 19606 genomic DNA with primers including Omp22-F CATGCCATGGCCATGCGTGCATTAGTTATT and Omp22-R CCGCTCGAGTTGTTTAGCATAAATGCT using PCR. The designed primers consisted of 5′-end *Nco*I and *Xho*I restriction sites, respectively. The optimized PCR program included pre-heating at 95 °C for 5 min, followed by 30 cycles of 1 min at 95 °C, 63 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplified gene *omp22* (654 bp) was purified by a gel purification kit (Thermo Scientific, Lithuania), digested, and cloned into the digested expression vector pET-28a+ (Novagen, USA) using T4 DNA Ligase (Thermo Scientific). The recombinant vector was transformed into *Escherichia coli* BL21 (DE3) competent host cells through the heat shock method and positive clones were confirmed by enzyme digestion. The expression was optimized by different concentrations of isopropyl-beta-thio galactopyranoside (IPTG) as an inducer [40]. Finally, the protein expression was evaluated by 15 % SDS-PAGE.

RESULTS

Protein Retrieval and Subcellular Localization

The complete sequence of the Omp22 contained 217 amino acids and the analysis by PSORTb v.3.0.2 online server and TMHMM Server v. 2.0 web tools showed the Omp22 as OmpA

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family protein belongs to the outer member proteins superfamily (with localization score of 10) without any transmembrane helices.

Antigenicity, Allergenicity Determination, and Similarity with the Human Proteome

The omp22 protein sequence was imported to VaxiJen, and the server confirmed the potential antigenicity of Omp22 with a high threshold value (0.74). Algpred analyses showed that Omp22 is a non-allergen. A comparison of the Omp22 sequence with the human proteome showed no significant similarity.

Physiochemical Characteristics of Omp22

Physiochemical characteristics were evaluated by the Expasy ProtParam server. Based on the results, the Omp22 molecular weight and isoelectric point parameters were determined as 22.48 kD and 9.30, respectively. The molecular formula of Omp22 was $C_{964}H_{1580}N_{288}O_{322}S_4$ and the net charge at pH 7.4 was estimated as +4.095**.** The estimated half-life of protein was over 10 hours in *E. coli*, *in vivo*, over 20 h in yeast, *in vivo*, and over 30 h in mammalian reticulocytes, *in vitro*. The instability index (II) is computed to be 30.23 as a stable protein. The aliphatic index and grand average of hydropathicity (GRAVY) were evaluated to be 84.61 and -0.288, respectively. The functional class analyses demonstrated that Omp22 is probably a metabolism molecule. The secondary structural content included 29% alpha-helix, 14% beta strands, and 8% disordered regions.

Identification of Linear and Conformational B-cells Epitopes and MHC Binding Sites

Two linear B-cell epitopes were identified in Omp22 (Table 1). Furthermore, the number of MHC-I and MHC-II binding sites was 108 and 50 peptides, respectively. None of the epitopes demonstrate any kind of toxicity *in silico*.

Table 1. Linear and conformational B cell epitopes of Omp22 protein of *A. baumannii.*

Linear B-cell epitopes	Start-end	Conformational B-cell epitopes	Score (for conformational B-cell epitopes)	Color
GNNLGGVEYDK	23-33	D150, N151, T152, G153, N154, D155, S156, I157, S190, D196, N197, S198, T199	0.84	Cyan
GISKSNANSSRQNNRA	47-62	D138, N139, K140	0.833	Hot pink
		N24, N25, L26, G27, G28, V29, E30, Y31, D32, K33, A34, A35, L36, K50, S51, N52, A53, N54, S55, S56, R57, Q58, N59, R61	0.815	Green

The 3D structure of Omp22 was successfully predicted and the quality of the tertiary structure was confirmed (Fig. 1A). ProSA-Web analysis of Omp22 3D structure showed a Z-score = -5.14 that confirms the similarity of the predicted 3D structure to the 3D structures of the other proteins that have been determined using X-ray crystallography or NMR spectroscopy (Fig. 1B). Moreover, the Ramachandran plot of Omp22 tertiary structure is shown in Fig. 1C. In a desirable 3D structure, > 90% of residues are located in favored and allowed regions of Ramachandran plot. After confirming the reliability of the 3D structure of the protein, we determined and showed the tertiary structure (Fig. 1D). Detailed information about the predicted conformational B-cell epitopes is presented in Table 1. In addition, the conservancy of the Omp22 protein was determined using ConSurf which shows the relative stability of Omp22 among *A. baumannii* strains (Fig. 2).

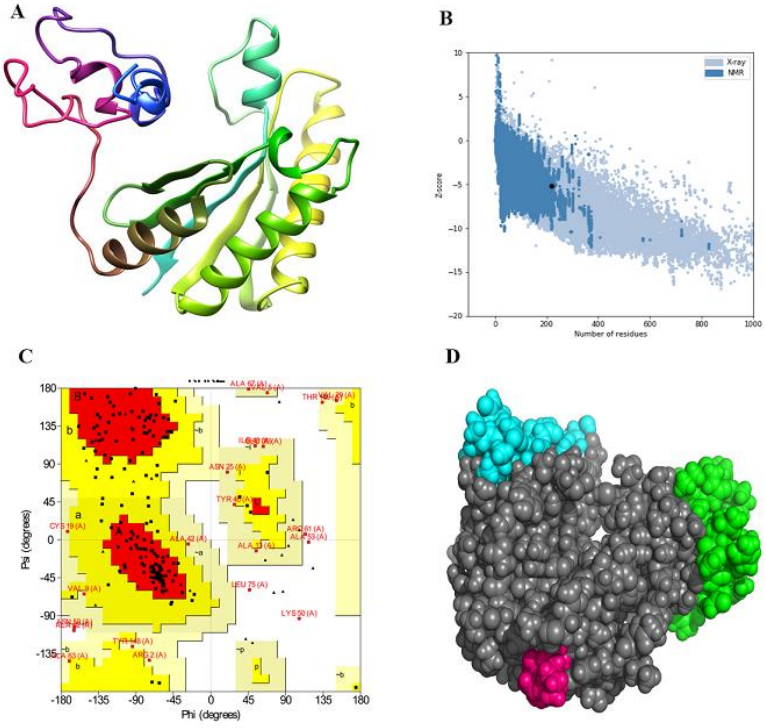

Fig. 1. A) The tertiary structure of Omp22 was predicted using the I-TASSER web server. **B)** ProSA-Web analysis of Omp22 3D structure showed a Z-score = -5.14. **C)** The Ramachandran plot of the Omp22 3D structure represents that over 90% of the residues were located in favored and allowed regions. **D)** The conformational B-cell epitopes of Omp22 (Cyan, hot pink, green) were identified using the ElliPro web server and demonstrated on the 3D structure of the protein

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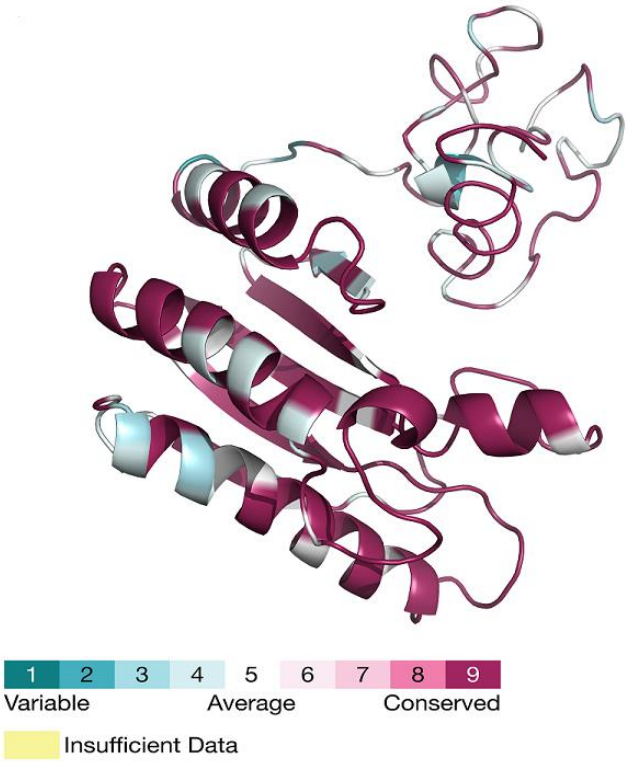
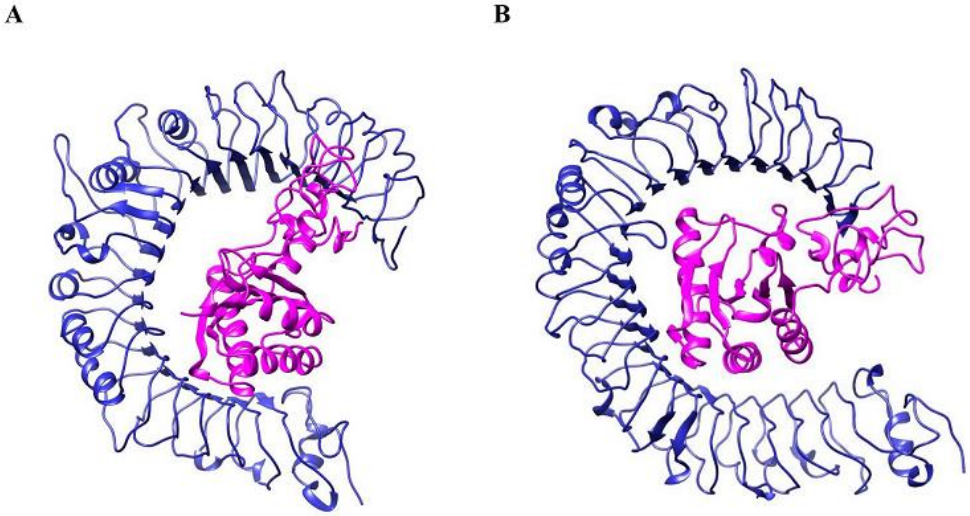


Fig. 2. Conservation of Omp22 was depicted using the ConSurf web server that confirmed the highly-conserved sequence of Omp22 among *A. baumannii* strains.

Molecular Dockings

Molecular docking of Omp22 and TLRs showed desirable interactions between compartments. The docking score of Omp22-TLR2 and Omp22-TLR4 docked complexes were -285.01 and -276.84, respectively (Fig. 3).



Docked complexes	Docking score	Confidence score	Ligand rmsd (Å)
A (Omp22-TLR2)	-285.01	0.9370	137.16
B (Omp22-TLR4)	-276.84	0.9267	84.20

Fig. 3. The molecular interactions of Omp22 and human TLRs were illustrated using the HDOCK web server and PyMOL software. **A)** Molecular docking of Omp22-TLR2. **B)** Molecular docking of Omp22-TLR4. The purple elements show Omp22 and the blue elements show human TLRs.

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Cloning and Expression Optimization of Omp22

The omp22-encoding gene (654 bp) from the *A. baumannii* genome was amplified, cloned into the expression vector pET-28a (+), and transformed into the expression *E. coli* BL21 (DE3) host cells by heat shock method. The positive clones were then verified by enzyme digestion (Fig. 4). The recombinant Omp22 (rOmp22) was then expressed under the control of a T7 strong promoter, as an IPTG-inducible promoter. The expression level of rOmp22 was evaluated by the addition of different concentrations of IPTG (ranging from 0.1 mM to 1 mM) and incubation times. Based on SDS-PAGE results, the optimized expression of the rOmp22 was successfully achieved with 0.5 mM IPTG for 4 h incubation at 37°C. The recombinant protein with a molecular weight of approximately 22 kDa is shown in Fig. 5.

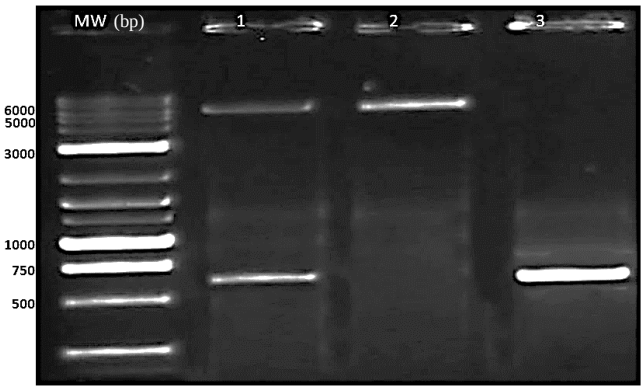

Fig. 4. Gene cloning confirmation with *Nco*I and *Xho*I enzyme digestion. MW: Molecular weight marker (1 kb ladder mix), Lane 1: Digested pET28a-*omp22* clone, Lane 2: Digested pET28a, and Lane 3: *omp22* gene (654 bp).

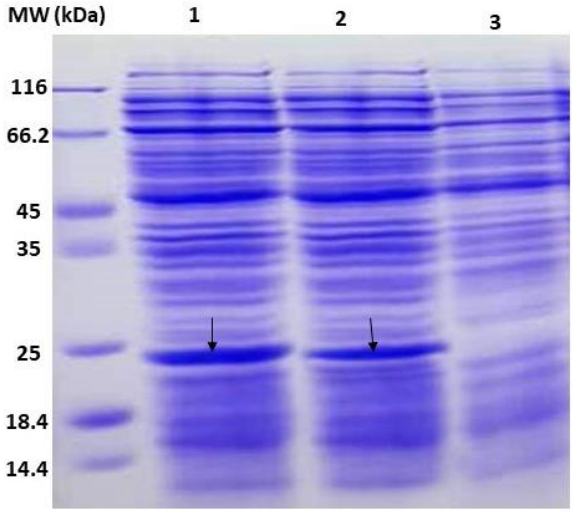

Fig. 5. Evaluation of Omp22 expression by SDS-PAGE. MW: Protein marker, Lane 1 and 2: induced clone (0.5 mM IPTG), and Lane 3: Uninduced clone. SDS-PAGE results showed that Omp22 has the appropriate expression at 0.5 mM concentration of IPTG.

DISCUSSION

A. baumannii is a major contributor to nosocomial infections and represents a significant threat to immunocompromised patients in healthcare settings. These patients are particularly susceptible to infections caused by *A. baumannii* due to the limited availability of effective treatment options, especially in the case of extensively resistant strains [41]. In this context, vaccination emerges as a promising strategy for the control of *A. baumannii* infections [42]. However, despite efforts in vaccine development, there is currently no suitable vaccine available against this pathogen. Therefore, extensive research is needed to identify and evaluate efficient vaccine candidates. Previous studies have identified several potential vaccine candidates with promising efficacy against *A. baumannii*. [43, 44]. It has been demonstrated that outer membrane proteins (OMPs) in *A. baumannii*, acting as porins and surface antigens, significantly contribute to the interaction and activation of the host immune response [45]. One such vaccine candidate, known as Omp22, has been identified from *A. baumannii*, demonstrating the ability to effectively stimulate immune responses in experimental animals [18].

Due to the limited available information regarding the functional characteristics of Omp22, this study aimed to assess Omp22 using bioinformatics tools and optimize its *in vitro* expression. Conducting *in silico* evaluations of vaccine candidates is a valuable approach that enables the assessment of potential efficacy and immunological characteristics, before proceeding to *in vitro* or *in vivo* experiments. This study sought to gain valuable insights into the efficacy of Omp22 as a potential vaccine candidate by employing bioinformatics tools [46]. Based on computational analysis, we found that Omp22 has several properties that make it a promising vaccine candidate against *A. baumannii.* In this regard, several analyses were performed to evaluate different aspects of the Omp22. The antigenic and allergenic properties of stable Omp22 were evaluated and the protein was predicted to be a non-allergenic antigen with a high threshold value.

The extracellular location, along with its non-allergenic and antigenic properties is one of the significant criteria for vaccine design [47]. The high aliphatic index (84.61) and the relative volume occupied by A, V, I, and L side chains in Omp22 serve as positive indicators of its thermostability [44, 48]. The low range Grand Average hydropathy (GRAVY) value (-0.288) indicates good interaction of Omp22 with water and has a polar nature [49, 50]. The Ramachandran plot analysis reveals that over 90% of residues in the chimeric structure are located in the favorable and permissible regions, with a low percentage in the outlier region. This suggests that Omp22 has suitable characteristics and acceptable quality of the protein model. This study suggests the presence of immunogenic B-cell and T-cell epitopes, which have the potential to stimulate both humoral and cellular immune responses.

These findings hold promise for the development of an epitope-based vaccine targeting *A. baumannii* infection. Synthetic peptides or epitopes can elicit immune responses due to their adaptability and specificity [51]. In this study, it was observed that a majority of the amino acids comprising the linear and conformational B-cell epitopes were hydrophilic and charged residues. This finding supports the notion that hydrophilic epitopes are highly immunogenic, particularly in the context of chimeric vaccine design [52]. The results showed an appropriate level of conservancy of Omp22 among *A. baumannii* strains. Choosing a protective candidate among different bacterial strains is important factor in vaccine design [23]. The interaction between immune cells and the vaccine plays a crucial role in establishing a durable immune response. Toll-like

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receptors (TLRs) are essential for pathogen recognition and contribute significantly to innate immunity [53].

To assess the molecular interactions between Omp22 and TLR2 or TLR4, molecular docking analysis was conducted. The docking scores of the Omp22-TLR2 and Omp22-TLR4 complexes were -285.01 and -276.84, respectively, indicating favorable binding affinities. TLR4 is expressed in monocytes, granulocytes, immature dendritic cells, and macrophage cells. The interaction between Omp22 and TLR4 or TLR2 promotes the activation of NF-κB, which in turn triggers immune responses [54]. In another phase of the study, we successfully cloned the *omp22*-encoding gene into expression vector pET-28a (+) for evaluation of Omp22 expression level in the *E. coli* BL21 (DE3). We analyzed different concentrations of IPTG and incubation times for finding the best expression conditions. Finally, we were able to optimize the expression of Omp22 by utilizing 0.5 mM IPTG and a 4h incubation at 37°C. This significant finding will greatly aid in conducting further studies on the development of subunit vaccines based on Omp22. In conclusion, based on the suitable immunoinformatic properties of Omp22 as well as expression feasibility, this study suggested that the Omp22 protein of *A. baumannii* could be applied to design an effective vaccine against *A. baumannii*. However, further experimental analyses are required**.**

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CONFLICT OF INTERESTS

The authors declare they have no conflict of interest.

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