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Epitope mapping of *Acinetobacter baumannii* outer membrane protein W (OmpW) and laboratory study of an OmpW-derivative peptide

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ABSTRACT

Outer membrane protein W (OmpW) is a less-known *A. baumannii* antigen with potential immunogenic properties. The epitopes of this protein are not well-identified yet. Therefore, in the present study, B- and T-cell epitopes of *A. baumannii* OmpW were found using comprehensive in silico and partially in vitro studies. The T-cell (both class-I and class-II) and B-cell (both linear and conformational) epitopes were predicted and screened through many bioinformatics approaches including the prediction of IFN-γ production, immunogenicity, toxicity, allergenicity, human similarity, and clustering. A single 15-mer epitopic peptide containing a linear B-cell and both classes of T-cell epitopes were found and used for further assays. For in vitro assays, patient- and healthy control-derived peripheral blood mononuclear cells were stimulated with the 15-mer peptide, Phytohemagglutinin, or medium alone, and cell proliferation and IFN-γ production assays were performed. The bioinformatics studies led to mapping OmpW epitopes and introducing a 15-mer peptide. In vitro assays to some extent showed its potency in cell proliferation but not in IFN-γ induction, although the responses were not very expressive and faced some questions/limitations. In general, in the current study, we mapped the most immunogenic epitopes of OmpW that may be used for future studies and also assayed one of these epitopes in vitro, which was shown to have an immunogenicity potential. However, the induced immune responses were not strong which suggests that the present peptide needs a series of biotechnological manipulations to be used as a potential vaccine candidate. More studies in this field are recommended.

1. Introduction

Acinetobacter baumannii is an oxidase-negative, catalase-positive, obligate aerobic, and non-motile bacterium that is found in abundance in nature and is usually isolated from soil, water, human skin, food, and sewage [1]. *A. baumannii* is known as an important pathogen due to its causing numerous infections and increasing resistance to various antibiotics [2]. The rate of isolation of *A. baumannii* from natural environments and its occurrence in the community is low, while the rate of its transmission to hospitalized patients is high. This bacterium usually causes illness in hospitalized patients and people with compromised immune systems [3]. This

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bacterium causes pneumonia, bacteremia, septicemia, soft tissue infections, wound infections, skin infections, urinary tract infections, endocarditis, and meningitis [4]. One of the most common clinical manifestations of *A. baumannii* infections is hospital-acquired pneumonia, and it mostly occurs in patients who are hospitalized in the intensive care unit and receive mechanical ventilation [5].

A. baumannii spreads easily, which is due to its ability to adhere to host cells, biofilm formation, and resistance to drying [6]. Accumulation of several resistance mechanisms in *A. baumannii* reduces the number of antibiotic classes available to treat infections caused by this bacterium [3,7]. It, therefore, underscores the importance of shifting the focus of the medical community from treatment to a vaccination strategy. Vaccination represents a promising and acceptable prophylactic strategy to prevent *A. baumannii* infection [8]. Several candidate vaccines have been tested to immunize model animals against *A. baumannii* infection, including whole-cell vaccines, polysaccharide vaccines, subunit protein vaccines, DNA vaccines, and multi-antigen vaccines [8–10]. So far, none of the *A. baumannii* vaccine candidates have been approved for use in humans, and efforts to find a suitable vaccine candidate are still ongoing.

Outer membrane proteins[1] (OMPs) are proposed as a suitable vaccine candidate for *A. baumannii*. OMPs are a group of bacterial proteins that play a decisive role in antibiotic resistance and pathogenicity of *A. baumannii* [11,12]. OMPs are abundantly found in the outer membrane of bacteria and often protrude from the polysaccharide capsule. OMPs usually elicit a strong immune response introducing them as suitable vaccine candidates [13–15].

Outer membrane protein W[2] (OmpW) is a 193 amino acid protein that is an important porin in the outer membrane of *A. baumannii* and many other bacteria [16–18]. OmpW is one of the most immunogenic proteins in *A. baumannii* and has a high ability to create an immune response in mice and is considered a good vaccine candidate for *A. baumannii* [19].

Epitopes are regions of an antigen that bind to antigen-specific membrane receptors on lymphocytes or to secreted antibodies. Immune cells recognize epitopes of an antigen. A single protein has many antigenic epitopes, but not all of these epitopes are capable to elicit a strong immune response. Epitopic peptide vaccines have a short sequence of amino acids and are composed of specific antigenic epitopes, which may create the appropriate immune response. These vaccines are very safe and affordable and may show higher antigenic activities than the parent antigen [20].

A. baumannii infections can be protected against through antibody-mediated immunity, as evidenced by successful passive immunization with monoclonal antibodies and the correlation of specific antibody titers with survival rates [19,21–23]. Vaccine development has largely focused on antibody-mediated protection, with less emphasis on cell-mediated protection [24]. The IgG isotype ratio has been used to assess Th1 or Th2-driven immunity, but few studies have investigated T cell responses [24]. Research has shown that inactivated or attenuated strains of *A. baumannii* can elicit both Th1 and Th2 responses, indicated by strong IgG1 and IgG2a (or IgG2c) antibody responses [21,25,26]. While the role of T cells in vaccine-induced protection remains unclear, some studies have examined T cell cytokine profiles and the impact of *A. baumannii* on antigen-presenting cells [27]. Further research is needed to determine the preferred T cell cytokine response for protection and the role of Th2 cells in providing B cell assistance [24].

Since the usual methods of finding effective epitopes in immunogenicity are time-consuming, expensive, and difficult, bioinformatics studies are used as a new way to develop vaccines through epitope prediction [28]. So far, no in vitro or bioinformatic studies for OmpW epitopes have been reported in information sources. The present study is one of the first studies in this field. In the current design, given both types of humoral and cell-dependent responses, B- and T-cell epitopes of *A. baumannii* OmpW were found using a comprehensive in silico study. Then the cellular immune responses against the best predicted epitopic peptide of OmpW were also evaluated by in vitro experiments.

2. Methods

2.1. OmpW amino acid sequence and 3D structure

The sequence of *A. baumannii* OmpW was found in the NCBI protein webserver (https://www.ncbi.nlm.nih.gov/protein) with the accession number AXV50845. Protein domain search was performed by the InterPro server (https://www.ebi.ac.uk/interpro/) and conserved domain search in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

The sequence was studied more by the BLASTP webserver (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), to find any conservancy in different organisms.

Since the structure of *A. baumannii* OmpW was not available in the PDB data bank (https://www.rcsb.org), its 3D structure was estimated by homology modeling using the SWISS-MODEL server (https://swissmodel.expasy.org/ [29]). The model's quality was checked by generating the Ramachandran plot using the MolProbity server (http://molprobity.biochem.duke.edu/ [30], and also by the SWISS-MODEL server-supplied scoring functions including qualitative model energy analysis[3] (QMEAN), local quality estimate[4] (LQE), and comparison with non-redundant PDB structures.

[1] - OMP: Outer membrane protein.
[2] OmpW: Outer membrane protein W.
[3] - QMEAN: qualitative model energy analysis.
[4] - LQE: local quality estimate.

2.2. T-cell epitope prediction and selection

Both 9-mer class-I ($CD8^+$) and 15-mer class–II ($CD4^+$) T-cell epitopes of OmpW were predicted by three prediction applications namely SYFPEITHI (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm [31]), IEDB (http://tools.iedb.org [32], and ProPred-I (https://webs.iiitd.edu.in/raghava/propred1/gloss.html) or –II (http://crdd.osdd.net/raghava/propred/), [33]. The prediction was performed using 10 and 7 highly frequent MHC alleles of HLA-I and HLA-II, respectively as reported in our previous studies [34–36].

The thresholds were used as the tools recommendations as follows: the percentile rank of ≤ 1 and ≤ 10 in IEDB for prediction class-I and –II epitopes, respectively; the tool's score of ≥ 10 for both epitopes' classes in SYFPEITHI and ProPred-I; and finally 4% of high-ranked class-II epitopes in ProPred-II.

Using the cluster analysis tool of IEDB (http://tools.iedb.org/cluster/) the obtained epitopes were clustered and those with more than 70% similarity were put in a unique cluster. Of each unique cluster, an epitope (preferably that with a higher prediction score) was selected and others were excluded.

The immunogenicity of class-I and the potential IFN-γ production of class-II epitopes was also estimated by the immunogenicity tool of IEDB (http://tools.iedb.org/immunogenicity/) and the IFNepitope webserver (https://webs.iiitd.edu.in/raghava/ifnepitope/application.php), respectively.

To find the regions of OmpW protein containing both class-I and –II T-cell epitopes, the shortlisted epitopes of both classes were compared and clustered by the IEDB cluster analysis tool with a threshold of $\geq 70\%$ similarity.

Finally, to make the predicted epitopes manageable numbers and to select the best (most immunogenic) epitopes, some criteria were taken as below: 1- The epitopic region containing both epitope classes, 2- The class-I or –II epitopes with higher scores taken from prediction tools, 3- The epitopes predicted by more tools, 4- The class-I epitope with higher immunogenicity score, and 5- The class-II epitope predicted positive for IFN-γ production.

2.3. Human similarity, allergenicity, and toxicity

The class-I and –II T-cell epitopes were predicted for possible similarity to human peptides, allergenicity, and toxicity. To evaluate any human similarity, the FASTA format of epitopes was entered into the BLASTP server, where the human was used as the organism and other parameters remained unchanged. The epitopes with $\geq 90\%$ coverage and simultaneously $\geq 90\%$ identity were excluded.

For allergenicity prediction, two tools were used including AllerCatPro [37] and Structural Database of Allergenic Proteins[5] (SDAP) [38]. For both tools, the default parameters remained intact. The toxicity of the epitopes was estimated by the ToxinPred server (https://webs.iiitd.edu.in/raghava/toxinpred/algo.php) [39] using the FASTA format of the epitopes as entry and remained the tool's parameters as default.

2.4. B-cell epitope prediction

Both linear and conformational B-cell epitopes were predicted as reported in our previous work [36]. Briefly, Antigenicity, Beta-turn, Flexibility, Hydrophilicity, Surface accessibility, Bepipred 1.0, and Bepipred 2.0 tools from IEDB (http://tools.iedb.org/main/bcell/), and the ABCpred tool (http://crdd.osdd.net/raghava/abcpred/ [40]) was used to predict the linear epitopes. The tools' defaults were selected as the prediction thresholds.

The predicted epitopes by each tool were screened and in case of a high number of predicted epitopes, the top ones (those with higher prediction scores) were selected. The shortlisted epitopes of all tools were then clustered by the IEDB cluster analysis tool and one epitope from each cluster (which was that with a higher score or predicted by more tools) was chosen as the final B-cell linear epitope.

Using the created model of OmpW as entry and leaving the tools' default intact, the conformational epitopes were predicted by two tools provided in IEDB namely DiscoTope 2.0 and ElliPro. The tools predicted one or more multi-segment conformational epitopes that were cleaned and recorded as final conformational epitopes.

2.5. Final 15-mer epitopic region (peptide)

Since conformational B-cell epitopes need accurate folding to be formed, they were not included in our final epitopic region. Otherwise, the final B-cell linear epitopes were compared with final class-I and –II T-cell epitopes. As a result, only one epitopic region (15-mer peptide) was found comprising a class-II (15 aa), a class-I (9 aa) T-cell epitope, and a core of a linear B-cell epitope (7 aa). This 15-mer epitopic peptide was selected for further molecular docking, physicochemical/structural analyses, synthesis, and in vitro assays.

5 SDAP: Structural Database of Allergenic Proteins

2.6. HLA-epitope molecular docking

The binding of the class-I and –II T-cell epitopes of the 15-mer peptide to their respective HLA molecules was assessed through molecular docking studies. The docking was run by HADDOCK 2.2 server (http://haddock.science.uu.nl/services/HADDOCK2.2) using tertiary structures of the epitopes (generated by PEP-FOLD 3.0 (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3)) and the HLAs 3D structures (obtained from PDB data bank). The residues involved in the binding were estimated by consensus prediction of interface residues in transient complexes (https://milou.science.uu.nl/services/CPORT) [41]. Applying these residues, the server run the HLA-epitope molecular docking and revealed the main results as the HADDOCK scores [42]. The original peptides (ligands) of crystallography-characterized HLAs in PDB files were used as control of the docking process.

2.7. Physicochemical/structural analyses of the 15-mer peptide

ProtParam tool (https://web.expasy.org/protparam/) [43] was used to estimate some physicochemical properties of the final 15-mer peptide including isoelectric pH[6] (pI), number of positively/negatively-charged amino acids, molecular weight (in Dalton unit), instability index, grand average of hydropathicity index[7] (GRAVY), and the estimated half-life. The PEPTIDE 2.0 tool (https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php) was used to estimate the peptide's hydrophobicity. The NovoPro Peptide Property Calculator (https://www.novoprolabs.com/tools/calc_peptide_property) was used to assess the peptide's net charge. Its 3D structure was assessed by PEP-FOLD 3.0 and depicted by the Web3DMol server (http://web3dmol.net/), the latter tool was also used to depict the peptide's location in the OmpW molecule 3D structure.

Using the. pdb file of the peptide, its secondary structures were predicted by the Stride Web interface (http://webclu.bio.wzw.tum.de/cgi-bin/stride/stridecgi.py). Finally, the possible conservancy of the peptide was evaluated by searching its sequence in the BLASTP server.

2.8. Peptide synthesis

The final peptide was sent to a commercial company (Peptron, South Korea) to be synthesized without any apparent modification. The synthesis was performed by an automatic peptide synthesizer (PeptrEX), confirmed by HPLC and Mass spectrometry methods (done by the company), and delivered to us in high purity (more than 80%) in Freeze-dried form (5 mg). The freeze-dried peptide was reconstituted in 1:3 v/v Acetonitrile/H_2O according to the manufacturer's instructions prior to its use in experimental tests.

2.9. Patients/controls selection and sample isolation

Five adult patients infected with *A. baumannii* infection for at least 7 days were identified in Imam Reza Hospital, Kermanshah city (west of Iran). Their gender, age, and type of disease were recorded from the medical files. The *A. baumannii* infection was confirmed in the hospital laboratory with culturing and biochemical methods. As controls, five age-matched healthy adults of both genders were selected.

From each participant, 15–20 ml of whole blood was taken and poured into collection tubes containing an anti-coagulant agent. The tubes were transferred to the research center for peripheral blood mononuclear cells[8] (PBMCs) isolation.

Written consent was obtained from all participants and the study was conducted under the supervision of the ethics committee of Kermanshah University of Medical Sciences (Code: IR. KUMS.REC.1399.1031).

2.10. PBMC isolation

The PBMCs were isolated using Ficoll-Paque as reported in previous works [44]. Briefly, the blood was centrifuged (30 min, 800×*g*) and the plasma (top layer) was taken out. An equal volume of PBS (pH 7.2) was added to the tube. Eight ml of the blood-PBS mixture was layered on top of 16 ml Ficoll-Paque and the tube was centrifuged (30 min, 400×*g*, room temperature) and the PBMC interface was collected by pipetting. The collected PBMCs were washed twice with PBS through two centrifugation steps (each 10 min, 100×*g*). The pellet was ready to be suspended in RPMI-1640 medium for culturing as below.

2.11. PBMCs culturing and stimulation

The fresh isolated PBMCs were cultivated in RPMI-1640 medium supplemented by FCS (10%) and Penicillin-Streptomycin, at 1–5 × 10^6 cells/ml for 24 h, at 37 °C. The cells were then counted and cultivated in 96-well plates at about 5000 cells/well (200 μl). The plate wells were assigned to PBMCs isolated from either patients or healthy controls. The peptide was added to the assigned wells at final concentrations of 10 μg/ml. The plate wells with Phytohemagglutinin[9] (PHA) addition (1% v/v) and without peptide/PHA were

6 pI: isoelectric pH

7 GRAVY: grand average of hydropathicity index

8 PBMCs: peripheral blood mononuclear cells

9 PHA: Phytohemagglutinin

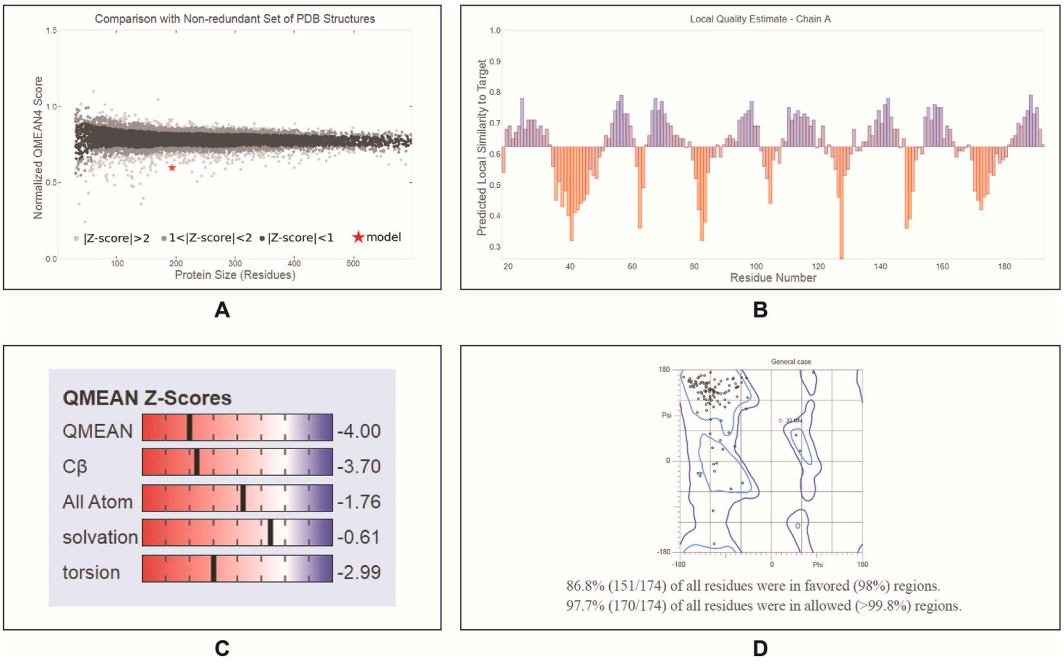

Fig. 1. The quality assessment of the OmpW model. After OmpW modeling by the SWISS-MODEL web server, the quality assessment of the model was performed by comparison with a non-redundant set of PDB structure (A), local quality estimate (LQE) (B), qualitative model energy analysis (QMEAN) scoring functions (C), and the Ramachandran plotting (D). The red star in the comparison plot shows the model, which its quality is high because it is near the grey region. In LQE, amino acids above 0.5 (the threshold) are of acceptable quality. In QMEAN scoring functions the dark lines (Z-scores) around 0 show a higher quality of the model. The high amount of amino acids in the allowed and favored regions of the Ramachandran plot shows the model's high quality. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

considered positive and negative controls, respectively.

The plate was then incubated in a CO_2 incubator (37 °C) for 96 h. Then, the contents of each well were divided into two portions, one was used for assaying cytokine responses, and the other was used for cell proliferation assay.

2.12. Cell proliferation assay by MTT assay

The cell proliferation assay was performed using MTT Cell Viability Assay Kit (DNAbiotech, South Africa). The procedure was performed according to the kit manual. Briefly, the MTT powder was dissolved in PBS and added to the plate wells (10 μl/well). The plate was incubated in the CO_2 incubator (37 °C) for 4 h. Then DMSO (100 μl/well) was added and the plate was incubated for a further 15 min with gentle shaking. Finally, the optical densities[10] (ODs) of the wells were obtained at 570 nm using an ELISA Plate Reader.

2.13. Cytokine response assay

For IFN- γ cytokine assay, a commercial kit (Karmania Pars Gene, Kerman, Iran) was purchased and the procedure was performed based on the kit manual. Briefly, the stimulated cells were centrifuged and the supernatants were collected for ELISA assay. In ELISA, the kit's standards and our samples were added to the assigned wells in a 96-well plate. The plate was incubated at room temperature for 1 h with mild shaking. After the washing step (5 times), the detection antibody was added and the plate was incubated for 1 h. Following a 5-times washing step, HRP-Avidin was added and the plate was again incubated for 30 min. Then the plate was washed and the substrate was added. After 15 min incubation, the stop solution was added and the ODs were read at 450 nm with an ELISA Plate Reader.

2.14. Statistical analysis

Student t-test was used to compare data between two groups, and the One-way ANOVA test was used to compare more than two groups with Tukey's test as multiple comparisons. $P \leq 0.05$ was considered as the significance level.

10 OD: optical density

Table 1
The top five T-cell epitopic regions of A. baumannii OmpW.

Epitopic region[b]	Class-II epitopes							
	Epitope	HLA allele No.[c]	Best score[a]					
			SYFPEITHI	ProPredII	IEDB	IFNg-induction	Toxicity	Allergenicity
VSS**FTFAGNWQV**KFG	VSSFTFAGNWQVKFG	5	30	–	1.7	–	–	–
AKFDL**DINPFVYTL**G	AKFDLDINPFVYTLG	4	10	–	0.73	+	–	–
KYHFK**NSTRFTPYI**G	KYHFKNSTRFTPYIG	4	38	+	0.6	+	–	–
NH**DVLLDGQKV**ARIK	NHDVLLDGQKVARIK	4	14	–	9.7	–	–	–
V**EYFFGQTPF**SAELL	VEYFFGQTPFSAELL	3	22	+	3.1	–	–	–

a- Only the highest score obtained is presented. Lower numbers in IEDB and higher numbers in SYFPEITHI and ProPredI indicate higher epitope prediction scores; In ProPredII only the epitope presence is indicated by "+".

b- Each row in this column shows a T-cell epitopic region containing a class-II epitope (whole sequence) and an internal class-I epitope (**bold underlined**).

c- The number of HLA molecules predicted to attach the epitope by prediction tools (sum of all tools' results) is presented.

3. Results

3.1. OmpW sequence and structure

Protein domain search showed that OmpW is comprised of 193 amino acids[11] (aa) residues with a large non-cytoplasmic domain from the superfamily of OMP/PagP_b-brl (InterPro code: IPR011250). Similarly, the conserved domain search in NCBI revealed a conserved domain of the protein belonging to the OmpW superfamily (Accession: cl43773).

Searching in the BLASTP showed that the protein is highly conserved among Acinetobacter species including *A. baumannii*, *A. lactucae*, *A. calcoaceticus*, *A. seifertii*, *A. nosocomialis*, *A. oleivorans*, as well as other bacteria including *Klebsiella pneumoniae* and *Escherichia coli*.

The 3D structure of OmpW was modeled by the SWISS-MODEL server with high quality as confirmed by the scoring functions of the server and the generated Ramachandran plot (Fig. 1).

3.2. T-cell epitope prediction

Initially, each tool predicted numerous epitopes (86, 405, and 27 class-I epitopes, and 135, 246, and 16 class-II epitopes by IEDB, SYFPEITHI, and ProPred, respectively). These epitopes were screened to find more probable and most immunogenic epitopes. These screening steps include deleting duplicate-predicted epitopes, choosing one of the much similar epitopes (preferably one with the highest prediction score), selecting those predicted by more tools, considering IFN-γ-induction by class-II epitopes, considering class-I epitopes with higher immunogenicity score, and finally removing the epitopes that were predicted to be an allergen, toxic, or similar to the human proteome.

These criteria led to the selection of the best T-cell epitopes as potential vaccine candidates. These epitopes were further compared, clustered, and five regions containing both epitope classes were found. All predicted parameters for these epitopic regions are presented in Table 1.

3.3. B-cell epitope prediction

The linear B-cell epitopes predicted by IEDB tools are presented as several plots (Fig. S1). These plots were compared and the shared epitopes between four or more tools were selected. These selected epitopes were then clustered with the ABCpred-predicted epitopes and an epitope (preferably that with a higher score or predicted by more tools) was selected as a more potential epitope. These final B-cell linear epitopes are presented in Table 2.

The DiscoTope 2.0 predicted a four-segment conformational epitope, while ElliPro predicted two conformational epitopes, each composed of several segments (Table 2). These epitopes are depicted in Fig. 2.

3.4. Molecular docking results and physicochemical/structural characteristics of the final peptide

By comparing the final linear B-cell and T-cell epitopes, only one epitopic region was found to possess both B and T (class-I and II) epitopes. This region, which consisted of a 15-mer peptide, was considered the final epitope. The molecular docking physicochemical/structural studies were performed for this peptide.

11 aa: amino acids.

Class-II epitopes	Class-I epitopes								
Best score[a]	Epitope	HLA allele No.[¥]	Best score[a]						
Human similarity (Coverage %, Identity %)			SYFPEITHI	ProPredI	IEDB	Immunogenicity	Toxicity	Allergenicity	Human similarity (Coverage %, Identity %)
93, 60	FTFAGNWQV	4	18	123	0.01	0.224	–	–	100, 57.1
88, 87.5	DINPFVYTL	7	28	–	0.03	0.159	–	–	88, 87.5
86, 43.7	NSTRFTPYI	5	21	–	0.06	0.204	–	–	100, 66.7
93, 72.7	DVLLDGQKV	5	17	–	0.7	−0.185	–	–	100, 70
90, 69.2	EYFFGQTPF	2	20	100	0.37	0.106	–	–	88, 85.7

Although the class-I and –II T-cell epitopes of the final 15-mer peptide attached to several HLAs, one HLA was selected for molecular docking studies; HLA-A*02:01 (ID: 5HHP) for class-I, and HLA-DRB1*15:01 (ID: 1BX2) for the class-II epitope. The tertiary structures of these HLAs were retrieved from the PDB webserver and used as receptors in the molecular docking study. The HADDOCK server conducted the dockings and revealed scores related to each complex. For both class-I-HLA and class–II–HLA complexes, the docking score was higher than controls (original peptide-HLA complexes) (Table 3).

Physicochemical characteristics of the final peptide showed that it is a stable and partially hydrophobic 1.86 kDa peptide with pI of 10, a net charge of 3.1, GRAVY of −1.047, and a high estimated half-life (Table 3). The peptide is mainly composed of turns and located on the surface of the OmpW protein structure (Fig. 2).

The peptide belongs to Moraxellaceae and is highly conserved among Acinetobacter species (Fig. S2).

3.5. Cases/controls characteristics

Five patients with different diseases but all infected by *A. baumannii* were included in this study. Their *A. baumannii* infection was confirmed by the hospital laboratory. Although the patients' characteristics may be important in immune responses, the present study only focused on the *A. baumannii* infection and neglected these characteristics. Otherwise, some demographic and medical characteristics of the patients were recorded and presented in Table 4.

3.6. Cell proliferation

The MTT assay showed that PBMCs were more proliferated in the presence of the 15-mer peptide than negative controls (non-peptide-added cells) but lower proliferated than the positive control (PHA) in four out of five patients, although only in three patients the differences were statistically significant (Fig. 3A). In one patient no difference was observed between peptide and the negative control. In contrast to the patients, only in one out of five healthy controls, the difference was observed between the peptide and the negative control (Fig. 3B).

Table 2
The top B-cell epitopes of *A. baumannii* OmpW.

Linear epitopes	Conformational epitopes					
	DiscoTope 2.0			Elipro		
	Start residue	Sequence	End residue	Start residue	Sequence	End residue
	Epitope #1			Epitope #1		
GGSVIAP	20	G	20	18	FAGNW	22
EDTTTAL	61	GQ	62	58	Y	58
KNSTRFT	103	KNSTRF	108	60	FGQTPF	65
KYHFKNS	147	PADAKN	152	100	Y	100
DEQGVAD				102	FKNSTRFTP	110
DKVKEDF				112	I	112
QPADAKN				145	FQPADAKNWGV	155
ADISPEVTLTNG				186	TLGYSYKF	193
QLPPTITA				Epitope #2		
YFFGQTPFSAELLLAT				36	DTTTALGVVKAD	47
				78	DVLLDGQKVAR	88
				122	WDEQGVADKVKE	133
				167	EVTLTNGAKFD	177

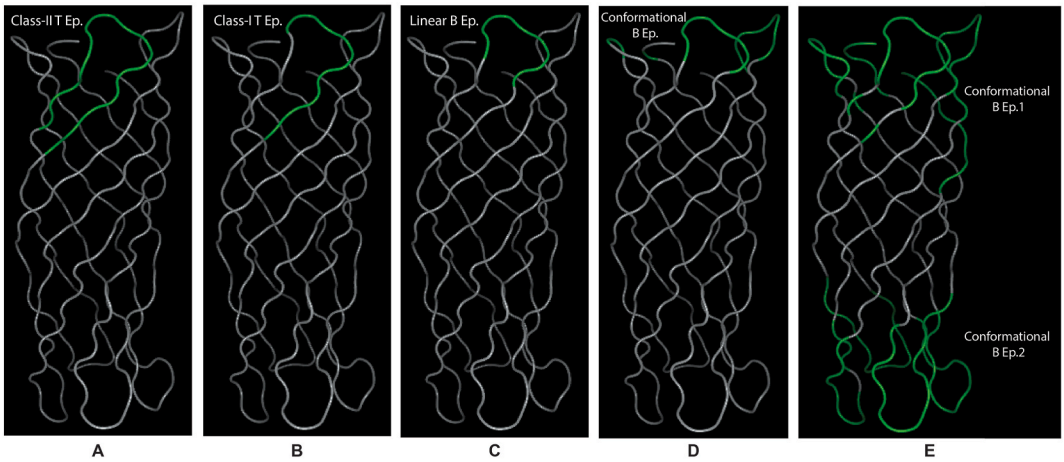

Fig. 2. The location of the final peptide, its T-cell and linear B-cell epitope, and the predicted conformational B-cell epitopes on *A. baumannii* OmpW 3D structure. The location of the final peptide (identical to the final class-II T-cell epitope) is shown in panel A, while the final class-I T-cell epitope is shown in panel B. The final linear B-cell epitope is shown in panel C. Predicted conformational B-cell epitopes by Discotope2.0 and Elipro are shown in panels D and E, respectively. The abbreviation "Ep." was used to refer to "epitope". In all panels, the epitopes are highlighted in green while the structure of *A. baumannii* OmpW is shown in grey. Note that in Elipro, two conformational epitopes were predicted and are shown as conformational B Ep.1 and conformational B Ep. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Cytokine production

The PBMCs of only one out of 5 patients significantly induced IFN-γ production following the peptide addition (Fig. 4A). Similarly, only one of the healthy controls' PBMCs induced IFN-γ production after the peptide addition (Fig. 4B). The PBMCs of all patients and healthy controls induced IFN-γ production after PHA addition (Fig. 4A and B).

4. Discussion

Although many efforts have been made to develop a suitable vaccine against *A. baumannii*, many of them have not resulted in final success. One of the important factors affecting the failure of *A. baumannii* vaccination and all other agents causing nosocomial infections is that these infections generally occur in hospitalized and mostly elderly people with weak immune responses [45]. It is more difficult to induce immune responses in these people than in healthy people [46]. It is assumed that such people who are susceptible to nosocomial infections are among the primary targets of vaccination, and therefore any vaccine candidate should be potent enough to elicit immune responses, alone or together with co-stimulatory molecules. This has caused researchers to always look for more suitable antigens as vaccine candidates against nosocomial infections, including *A. baumannii*.

Table 3
The final 15-mer epitopic peptide of *A. baumannii* OmpW.

The peptide sequence	KYHFKNSTRFTPYIG
Class II T-cell epitope (selected HLA-II)	KYHFKNSTRFTPYIG (DRB1[a]01:15)
Class I T-cell epitope (selected HLA-I)	NSTRFTPYI (A[a]02:01)
Linear B-cell epitope	KNSTRFT
Docking score class-II epitope-HLA-II (control)[a]	−70.8±-11.6 (−67.3±-10.1)
Docking score class-I epitope-HLA-I (control)[a]	−87.1±-10.9 (−69.2±-13.3)
Number of amino acids	15
Molecular weight	1.86 kDa
Net charge at pH = 7.0	3.1
Grand average of hydropathicity index (GRAVY)	−1.047
Hydrophobicity	26.67%
Isoelectric pH (pI)	10
Instability index	0.63, stable.
Estimated half-life:	1.3 h (mammalian reticulocytes, in vitro) 3 min (yeast, in vivo) 3 min (*Escherichia coli*, in vivo)
Secondary structure	KYHFKNSTRFTPYIG
E = Strand, T: turn	.EETTTTTTEE … ….

[a] - The docking scores are presented as HADDOCK score±standard deviation. More negative scores represent higher binding affinity. Controls are the original ligands of crystallography-characterized HLAs in PDB files.

Table 4
The characteristic of the patients included in the present study.

Patient No.	Age (year)	Gender	Hospitalization	Diagnosis	Ward	Infectious agent	Detection method	Matched healthy control
1	58	Female	Yes	Burning	Burn	*A. baumannii*	Bacterial culture, Biochemistry	A 57-year-old woman
2	86	Female	Yes	Stroke	Stroke care unit	*A. baumannii*	Bacterial culture, Biochemistry	A 66-year-old woman
3	71	Male	Yes	Loss of consciousness	Brain intensive care unit	*A. baumannii*	Bacterial culture, Biochemistry	A 53-year-old man
4	41	Male	Yes	Emphysema	Intensive care unit	*A. baumannii*	Bacterial culture, Biochemistry	A 41-year-old man
5	72	Male	Yes	Urinary tract obstruction	Kidney and urinary tract	*A. baumannii*	Bacterial culture, Biochemistry	A 62-year-old man

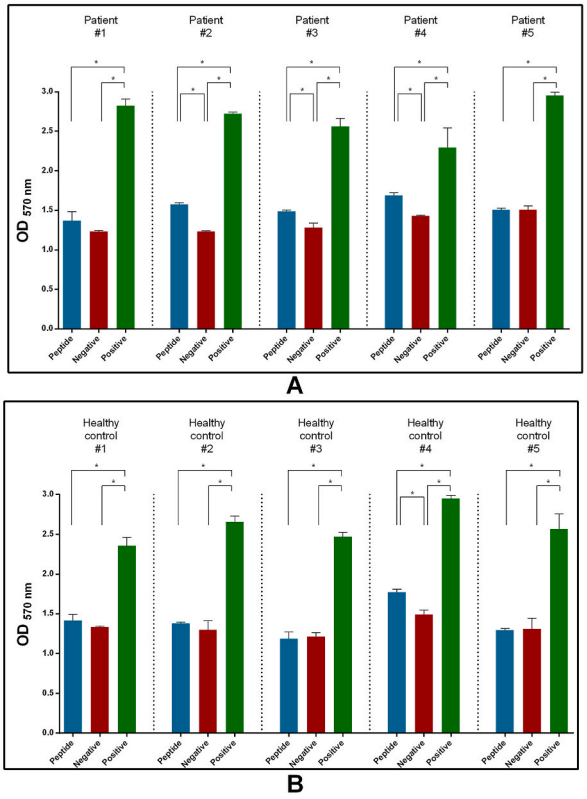

Fig. 3. The result of PBMCs proliferation assay. A) The PBMCs of patients no.2, 3, and 4 were significantly more proliferated in the presence of the final 15-mer peptide (blue bars) than wells without peptide (Negative) (red bars), but lower proliferated than wells with PHA addition (Positive) (green bars). In patients no.1 and 5 no statistically significant difference was observed between peptide and the negative control. B) In healthy controls, only in healthy control no.4 a significant difference was observed between the peptide and the negative control. The statistical differences between groups are shown by star symbols (p-value≤0.05). Each bar represents the mean value of three experiments per group, with error bars indicating the standard deviation of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In the current project, we found the most effective parts (epitopes) of the OmpW antigen from *A. baumannii* by using bioinformatics potential. A large number of epitopes were predicted. Since the examination of all these epitopes requires spending a lot of money and time, it was logical to identify the best of them and focus on them. To select this best epitope, bioinformatics was also very helpful, and by these methods, we identified a 15-mer epitopic peptide. This 15-mer peptide contains a class-II, a class-I T-cell epitope, and a core of a linear B-cell epitope. Therefore it was expected to elicit cell-dependent (both cytotoxic and helper T-cell) and humoral responses. This peptide was estimated to be immunogenic, able to induce IFN-γ, bind to HLAs with high affinity, and not be toxic or allergenic to humans. The investigation of the physicochemical properties of this peptide showed that this peptide is stable and has a relatively suitable half-life. Also, its pI is far from the physiological pH of the human body, thus the possibility of its precipitation in the body is low [47]. However, the peptide is not very soluble in water and its dissolution required relatively non-polar solvents. Although this

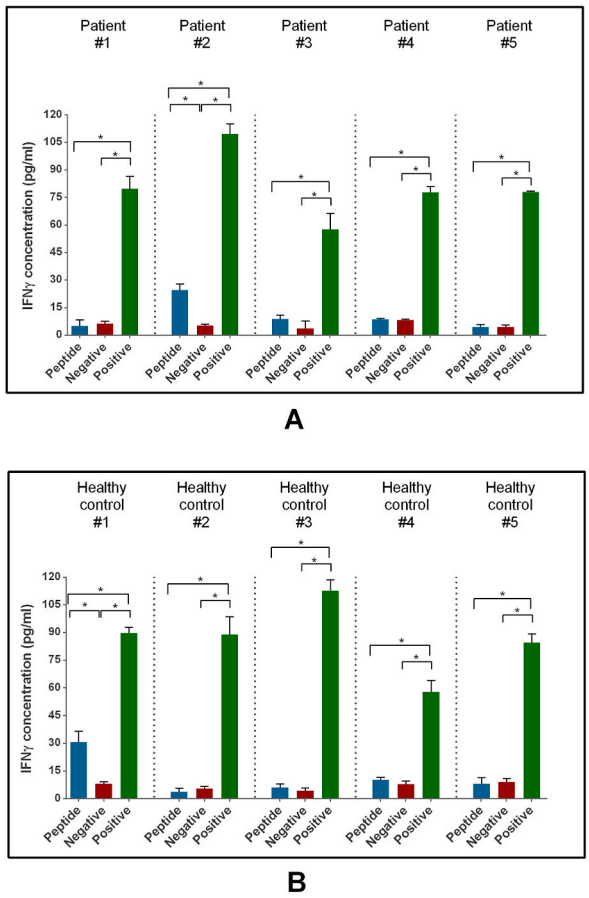

Fig. 4. Cytokine production assay results. A) The PBMCs of patient no.2 significantly induced IFN-γ production following the peptide addition. B) The PBMCs of healthy control no.1 induced IFN-γ after the peptide addition. The PBMCs of all patients and healthy controls induced IFN-γ production after PHA addition (Positive, green bars). Negative bars (red bars) indicate the wells without peptide addition. Each bar represents the mean value of three experiments per group, with error bars indicating the standard deviation of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

issue is not ideal for a vaccine candidate [48], it can be overcome by structural changes in the peptide, connecting it to a suitable adjuvant, or transporting it by carriers such as liposomes [49].

Although the prediction of epitopes by bioinformatics greatly reduces the time and cost of finding epitopes, whatever it is, it is considered a type of prediction and its authenticity should be confirmed by laboratory methods [50]. For this reason, in the present project, PBMCs from people infected with *A. baumannii* were used to investigate the potential immunogenicity of the epitope peptide obtained from our bioinformatic evaluations. This strategy has already been used in the vaccination of other pathogens [51–55]. However, the challenges facing nosocomial infections to adopt such a strategy are more that some of them are discussed in the following lines.

One of the most important criteria for the effect of a peptide on cells is its effect on proliferation. An appropriate immunogenic peptide may be presented to T cells in the aggregate of PBMCs, stimulate them, and increase their proliferation. Although these responses are also seen in first exposure to the antigens, the strength and speed of them are more evident if there are memory T cells [56]. Since patients with *A. baumannii* infections have already been in contact with this pathogen, it is expected that they have given an immune response against it and have created memory cells in a period of at least 7 days [57]. In our study, cells from 3 (out of 5) patients and one (out of 5) healthy controls showed significantly stronger proliferative responses than the negative control. These results indicate the validity of choosing the peptide as an immunogenic vaccine candidate. However, the proliferative rate was not very high and there were some individuals (2 patients and 4 healthy controls) who did not respond to the peptide. This may downgrade the potency of our peptide as a proliferative agent, but there are many neglected parameters that may cause this result. Of these parameters, the following can be mentioned: 1- The lack of accurate *A. baumannii* identification, 2- Lacking enough time to build memory cells, 3- Being old and the overall low ability to induce an immune response, 4- Lacking the HLAs which can bind to the peptide, and 5- the experiment-related errors of our study.

The potential role of immunosenescence in the observed low immunogenicity of the epitope candidate should be explored further in future studies. Additionally, examining the immunogenicity of overlapping peptides spanning the entirety of the OmpW protein may

provide valuable insights into the design of more effective epitope-based vaccines. Also, it is suggested that to induce a strong proliferative response, the peptide be combined with co-stimulatory molecules. Toll-like receptor agonists[12] (TLRs) can be mentioned among the molecules that help in inducing immune responses [58,59]. These molecules can be used as adjuvants and promote immune responses. The ability of these types of TLRs agonists as adjuvants for vaccination against various pathogens has been reported in several studies [60–64].

In addition to humoral responses that are effective in protecting against *A. baumannii* infection, cellular responses can also play a significant role in protecting against this pathogen, although there are conflicting opinions in this regard [24]. In any case, these responses usually fight the infectious agent by inducing cytokines in addition to increased proliferation. Although many cytokines may play a role in these responses, it has been shown that IFN-γ, as the main representative of type 1 helper[13] (Th1) responses [65], plays an important role in fighting against infections. Therefore, this cytokine was investigated in the present study. The results showed that the cells of only one patient and one healthy control were able to increase IFN-γ secretion after exposure to the peptide. Although the present study used a time interval of 96 h (4 days) after stimulation for cytokine assays, which was based on some studies [53,54], it seems that for the induction of IFN-γ, a longer time (at least 10 days) and even re-stimulation with peptide and/or with other co-stimulatory molecules (such as IL-10) are required [66]. In addition, the non-responsive of cells in IFN-γ production may be also due to the above-mentioned reasons for the non-responsive of cell proliferation.

It should be noted that this study only investigated the possibility of identifying this peptide by patients' cells and the possibility of using it as a vaccine requires much more studies. There are some limitations of the present study. First, the sample size of our study is small. However, this was largely out of necessity, because we need old *A. baumannii*-infected patients with long-time hospitalized (at least 7 days) or discharged and need to back to the hospital for sampling, without other infection than *A. baumannii*, and willing to participate in our study. Therefore, it was very difficult to increase the sample size. Second, the study includes the lack of accurate matching of cases and controls, although this is very difficult and the only parameters we were able to use for matching were the gender and to some extent the age of the people. Third, despite the identification of potential B-cell epitopes in silico, we were not able to examine their potency in vitro due to the preliminary nature of this study. Therefore, the effectiveness of these epitopes in inducing a humoral response could not be determined. Future studies may investigate the immunogenicity of the identified B-cell epitopes by examining their potency in vitro using techniques such as ELISA and western blotting. Fourth, the study includes not take into account the background (especially the previous exposure to nosocomial infectious agents) and many personal and medical factors of the patients and healthy control, and their possible effect on the amount and intensity of the responses. Noteworthy, there are many more parameters for a vaccine to be successful in the real world, which can be investigated in the form of further primary studies on animal models. Among these parameters, we can mention the appropriate amount of antigen, injection route, injection dose, vaccination schedule, adjuvants, etc. [67,68].

In general, in the current project, we mapped the most immunogenic epitopes of OmpW that may be used for future studies and also assayed one of these epitopes in vitro, which was shown to have an immunogenicity potential. However, the induced immune responses were not strong and this shows that the present peptide needs a series of biotechnological manipulations such as attachment to adjuvant or use in the form of multivalent or multi-epitope molecules to be used as a vaccine. More studies in this field, particularly on possible humoral responses evoked, are recommended.

Author contribution statement

Hana Heidarinia: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Elahe Tajbakhsh: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, Analysis tools or data.

Mosayeb Rostamian: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hassan Momtaz: Performed the experiments.

Data availability statement

Data included in article/supp. Material/referenced in article.

Additional information

Supplementary content related to this article has been publish online at [URL].

Ethics approval

The study was conducted under the supervision of the ethics committee of Kermanshah University of Medical Sciences (Code: IR.KUMS.REC.1399.1031).

12 TLR: Toll-like receptor agonist

13 Th1: type 1 helper

Consent to participate

Written consent to participate was obtained from all participants.

Consent to publish

Written consent to publish was obtained from all participants.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18614.

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