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In Silico Design and Evaluation of *Acinetobacter baumannii* Outer Membrane Protein A (OmpA) Antigenic Peptides As Vaccine Candidate in Immunized Mice

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ABSTRACT

Acinetobacter baumannii is a Gram-negative bacterium that has recently been identified as a leading nosocomial pathogen. Infections by this pathogen result in significant mortality due to antibiotic resistance. An effective vaccine would help alleviate the burden of disease incurred by this pathogen; however, there are currently no licensed vaccines offering protection against *Acinetobacter baumannii* infection.

In this study, considering the fact that outer membrane protein A is one of the most promising vaccine candidates, we predicted T cell and B cell epitopes on this protein using sequence-based epitope prediction tools and determined whether or not mice immunized with these peptides induce an immune response. We selected consensus epitopes including five peptides in different tools with the highest score.

48 female C5BL/6 SPF injected subcutaneously with the peptides (peptide1 to peptide 5 separately) in 100 µL of the solution and sham groups received adjuvant and PBS alone on the same schedule: on day 0 (primary dose) and two booster doses were administered on days 14 and 28. At the end of time, animals euthanized by Isoflurane, and collected sera for assessment of specific antibodies against each peptide by ELISA (Enzyme-linked immunosorbent assay).

Immunization of mice showed one of the novel synthetic peptides (peptide 1 (24-50 amino acids)) elicited immune responses. We conclude to combine theoretical methods of epitope prediction and evaluating the potential of immunogenicity for developing vaccines is important.

Keywords: *Acetinobacter baumannii*; Antigenic peptide; In silico; Outer membrane protein A (OmpA); Vaccine

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INTRODUCTION

Acinetobacter baumannii is known as an emerging pathogen responsible for several serious nosocomial infections, including Ventilator-Associated Pneumonia (VAP); which may progress to septicemia in the intensive care unit (ICU) patients.[1] Other clinical manifestations of *A. baumanii* include secondary meningitis, as well as bloodstream, wound, urinary tract, and burn infections.[2]

The antibiotic resistance of *A. baumannii* is frequently responsible for the failure of antibiotic therapies; therefore, vaccines to prevent multidrug-resistant *A. baumannii* infections are needed.[3] Experimental and *in silico* analyses have demonstrated which one potential vaccine candidate that may prevent these infections is *A. baumannii* outer membrane protein A (OmpA).[4] OmpA acts as an important virulence factor as it is reported to play critical roles in biofilm formation, immune stimulation, adherence to and invasion of eukaryotic cells, apoptosis, outer membrane vesicle biogenesis, and serum resistance.[5] Comparison of OmpA among other sequenced strains have shown that OmpA is highly conserved (>80%) and has minimal homology to the human proteome. Since, OmpA induces strong immune responses, making it an ideal candidate for vaccine development.[6] Among different Antigens screened, OmpA was identified as a target that it can stimulate humoral immune responses. Anti OmpA Antibodies increase protection through enhanced opsonic-phagocytosis.[1]

Epitope prediction is the process by which epitopes induce strong immune responses that are selected for the production of epitope-based vaccines and diagnostics methods. The advantages of epitope-based vaccines over traditional methods include reduced cost, complexity, and synthesis time.[7] Current epitope-based vaccines include both B and T cell epitopes. Indeed, identification of both types of epitopes will likely be necessary for effective epitope-based vaccine design. Although some of these epitopes will possess limited immunogenicity, this problem can be addressed by combining them with immunogenic adjuvants or carrier proteins.[8]

In the current study, we focused on OmpA as a target antigen for the sequence- and structure-based epitope prediction in order to design a vaccine that is protective against *A. baumannii* infections. We first determined the OmpA protein sequence and then combined bioinformatics strategies and immunization of mice to identify immunoreactive B and T cell linear epitopes and evaluating the immunogenicity of synthetic peptides in mice respectively.

MATERIALS AND METHODS

The current study is divided into two sections. In the first section, we determined the *A. baumannii* OmpA nucleotide and amino acid sequences of the bacterial strain isolated from patients. In the second section, we analyzed OmpA protein for B and T cell epitopes in order to determine immunoreactive peptides in mice. All experiences were carried out in compliance with the requirements of the animal ethical committee at Shahid Beheshti University of Medical Sciences (Ethical Approval NO. 8223, February 2, 2016).

Bacterial Growth Conditions

A. baumannii was isolated from Bronchoalveolar lavage (BAL) samples from hospitalized pneumonia patients in the intensive care unit (ICU) of Masih Daneshvari hospital in Tehran, Iran (we had served consent form before collecting samples).[2,9] *A. baumannii* was grown in Luria Bertani (LB) broth and on nutrient agar culture medium for 24 h at 37°C.

Gene Amplification

OmpA sequence of *A. baumannii* genomic DNA (extracted by Roche kit, Germany) was amplified by polymerase chain reaction (PCR). The OmpA specific primers used in the amplifications are ATGAAATTGAGTCGTATTGC (forward primer) and TTATTGAGCTGCTGCAGG **(**reverse primer), the primers were designed and ordered by Sinaclon Company. DNA was amplified using the following conditions: one cycle of 95°C for 10 minutes, 30 cycles of 95°C for the 30s, 50°C for 45s, and 72°C for 45s, and one cycle of 72°C for 10 minutes. The full-length PCR product was sequenced and translated into an amino acid sequence by the EMBOSS Transeq server. In the next stage, we aligned this OmpA sequence and other *A. baumannii* OmpA sequences using the multiple sequence alignment tool FlorenceCorpet.[10]

Secondary and Tertiary Structure Prediction and Analysis

To predict secondary structure, we used the GOR4

(Garnier-Osguthrope-Robson) server to identify alpha helices, beta strands, turns, and random coils. OmpA structure was also modeled by the I-TASSER (Iterative Threading Assembly Refinement) server.[11,12] I-TASSER is a hierarchical approach to protein structure and function prediction. Structural templates are first identified from the Protein Data Bank by a multiple-threading approach using LOMETS (Local Meta-Threading Server), full-length atomic models are then constructed by iterative template fragment assembly simulations. Finally, target functions are determined by threading the three-dimensional models through the protein function database BioLip. Thus, I-TASSER produces results by template-based homology modeling. To verify the predicted model, the Ramachandran plot was analyzed with PROCHECK in the PSVS server 1.5.[13]

Prediction of B and T Cell Epitopes

To identify potential OmpA antigenic epitopes, physicochemical properties including hydrophilicity, antigenicity, flexibility, mobility, accessibility, polarity, exposed surfaces, and coils were analyzed. We used several tools to predict epitopes based on these properties. To predict linear B cell epitopes, we used the following tools: LBtope, with an overall accuracy of 81%;[14] SWMTrip, with sensitivity and precision of 80.1% and 55.2%, respectively;[15] ABCpred, with sensitivity, specificity, and positive prediction of 67.14%, 64.71%, and 65.61%, respectively;[16] Kolaskar and Tongaonkar antigenicity prediction with accuracy 75%; BcePred, with a prediction accuracy between 52.92% and 57.53%;[17] BepiPred, with 75% accuracy;[10] and BCPred, with classifier specificity of 75%.[18] We also used CBTope server to predict conformational epitopes based on the primary sequence with 86.59% accuracy.[19]

Predicted Linear B cell epitopes were then analyzed to detect the presence of T cell epitopes using the MHC-II Binding Predictor in the immune epitope database (IEDB) with a specific focus on epitopes that bind to H2 I-A_b alleles related to C57BL/6 mice. Positive T cell epitopes were identified by an H2-binding prediction score of less than 30.[20]

Computation of Physical and Chemical Parameters of Epitopes

ProtParam was used to compute various physical and chemical parameters. These parameters included theoretical isoelectric point (pI), instability index, estimated half-life, aliphatic index, and grand average of hydropathicity (GRAVY). We then selected peptides based on these parameters. Finally, our selected peptides were synthesized by the 1ST BASE Company.

Mice Immunization

Female 6-8 week old C57BL/6 mice (6 animals per group) were purchased from the animal facility, Shahid Beheshti University of medical science. Mice were checked for specific pathogen-free (SPF) before the beginning of immunization So that inclusions criteria include factors like age (6-8 week old), sex (female) and SPF mice. We used SPF mice to demonstrate mice were free of a specific list of pathogens by routine testing. The list of organisms assessed typically includes disease-causing pathogens that can affect mouse health and research outcomes, as well as opportunistic organisms that typically do not cause illness in normal, healthy mice. All mice have been housed in sterilized cages. All supplies including food, water, and bedding introduced into an isolator room had been sterilized; the most frequently used sterilization method was autoclaving for 15 min at 121°C. As the diet might harden and become difficult to gnaw after autoclaving, the use of gamma-irradiated food. We did routine tests for the culture of bacteria before starting immunization and made sure they were SPF. The peptide (50μg) was suspended in PBS (Phosphate Buffered Saline) and emulsified in a 1:1 ratio of Aluminum hydroxide gel adjuvant (InvivoGen, USA). The animals were divided into 8 groups (Table 1) and injected subcutaneously behind of neck with the peptides (peptide1 to peptide 5 separately) in 100 μL of the solution and sham groups received adjuvant and PBS alone on the same schedule. The mice were inoculated with vaccine on day 0 (primary dose) and two booster doses were administered on days 14 and 28. We oversaw the basic animal maintenance including housing, a palatable diet, water, and bedding. Two weeks after the last injection, sera were collected and stored at -20°C for further use and then animals were euthanized by Isoflurane.

ELISA

ELISA assay was adopted for the detection of antibodies against peptides. ELISA plates were coated with 100 μL per well of 5 μg of each peptide. The coated wells were blocked with bovine serum albumin,

incubated with mouse sera, washed, and then stained with rabbit anti-mouse secondary antibody IgG H&L conjugated with horseradish peroxidase (Abcam, USA).

Table 1. Groups of mice PBS (phosphate buffered saline)

Mouse group no.	Immunization treatment
1	Control
2	PBS (sham)
3	PBS* + Adjuvant (sham)
4	Peptide 1+Adjuvant
5	Peptide 2+Adjuvant
6	Peptide 3+Adjuvant
7	Peptide 4+Adjuvant
8	Peptide 5+Adjuvant

The wells were washed again and incubated with 3,3',5,5'-Tetramethylbenzidine substrate (SIGMA-ALDRICH, USA). The color was allowed to develop for 20 minutes upon the reaction was terminated by adding 2N sulfuric acid (50 µL) and the Optical Density (OD) was determined at 450 nm in a microtiter plate reader (Multi-mode Reader, BioTek).

Statistical Analysis

Analysis of all of the data was performed by SPSS 25 software (SPSS software version 25, Inc, Chicago, IL, USA). Antibody titers were compared; using Kruskal-Wallis test. A *p*-value of smaller than 0.05 was considered as the threshold of statistical significance for all of the parameters.

RESULTS

OmpA Sequence

The full-length OmpA gene was amplified by the PCR method (Figure 1) and sequenced. The 356-amino acid OmpA protein sequence was determined from the nucleotide sequence and aligned with other OmpA protein sequences in the National Center for Biotechnology Information (NCBI) database (Figure 2). The sequences are highly homologous. The NCBI database identification number for the OmpA protein analyzed in this study is ATG88079.

Prediction of OmpA secondary and tertiary structures and confirmation of the modeled structure:

The OmpA protein secondary structure was analyzed for alpha helices, beta strands, turns, and random coils. The epitopes were almost located in coils (Figure 3). To predict B cell epitopes, the modeled tertiary structure was analyzed with PROCHECK, which predicted that 79% of OmpA residues are in favored regions, 17.4% in additional allowed regions, 2.7% in generously-allowed regions, and 0.9% in disallowed regions (Figure 4).

Prediction of B and T Cell Epitopes in OmpA Protein

After determining the OmpA amino acid sequence, we predicted the protein's antigenic peptides. Based on hydrophilicity, antigenicity, flexibility, mobility, accessibility, polarity, exposed surface, and coils, five OmpA epitopes were selected using the web-accessible B cell prediction servers LBtope, SWMTrip, ABCpred, BepiPred, IEDB, BCPred, BcePred, and CBTOPE. When selecting epitopes, we excluded the 23-amino acid N-terminal signal peptide using SignalP 4.1. Top epitopes were selected based on scores, repeats, and overview of the consensus from the various Linear B cell prediction servers with a specific focus on epitopes that bind to H2 I-A_b alleles related to C57BL/6 mice. We also selected epitopes based on secondary and tertiary structure analyses (Table 2). Finally, we considered biochemical properties for the selection of peptides. Different properties including the theoretical pI, estimated half-life, instability index, aliphatic index, and GRAVY were examined (Table 3).

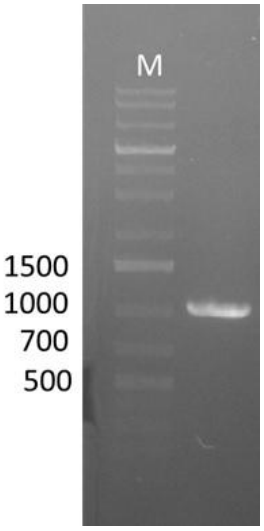

Figure 1. Agarose gel electrophoresis of outer membrane protein A (OmpA) PCR products: *A. baumannii* genomic DNA was isolated, amplified by PCR using OmpA specific primers, and electrophoresed on the gel. Lane M: molecular weight marker (1kb plus), lane (1071 bp)

Response to Immunization by Peptides

In order to evaluate the antibody response in mice immunized by designed peptides, the sera collected were used in ELISA to assess the reactivity of antibodies produced by immunization against the peptides. The results demonstrated that serum reacted with one of the peptides (24-50 amino acids) present in the ELISA plate is significant (p value<0.05). We also checked serum of control group without any injections and serum collected from mice immunized with PBS (sham) as well as immunized mice with PBS plus adjuvant (sham), did not react with these peptides. We investigated the sera of control, PBS (sham), and PBS plus adjuvant (sham) groups with each peptide separately (Figure 5).

Figure 2. Alignment of multiple outer membrane protein A (OmpA) protein sequences registered in the national center for biotechnology information (NCBI) database. ATG88079 is our registered protein in NCBI.

Figure 3. Prediction of outer membrane protein A (OmpA) protein secondary structure using GOR4.

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Table 2. B and T cell epitopes of outer membrane protein A (OmpA) were predicted by various servers based on physicochemical properties. Overlapped B and T cell epitopes are shown together.

Peptides	Sequence	Length	Position of Amino acids
Peptide 1	VTVTPLLLGYTFQDSQHNNGGKDGNLT	27	24-50
Peptide 2	YVLLGAGHYKYDFDGVNR	18	122-139
Peptide 3	LRVFFDTNKSNIKDQY	16	230-245
Peptide 4	ADNKTKEGRAMNRRVF	16	317-332
Peptide 5	HLKPAAPVVEVAPVEPTPVAPQPQEL	26	197-222

Table 3. Physical and chemical parameters of outer membrane protein A (OmpA) epitopes include theoretical Isoelectric point (pIs), instability index, estimated half-life, aliphatic indexes, and grand average of hydropathicities (GRAVYs)

Peptides	pI	HALF-LIFE	Instability index	Aliphatic index	GRAVY
Peptide 1	5.2	100h	24.51	79.2	-0.49
Peptide 2	6.7	2.8h	44.96	81.11	-0.36
Peptide 3	8.5	5.5h	42.58	66.8	-0.9
Peptide 4	10.9	4.4h	54.67	30.63	-1.49
Peptide 5	4.75	3.5h	92.3	101.15	-0.02

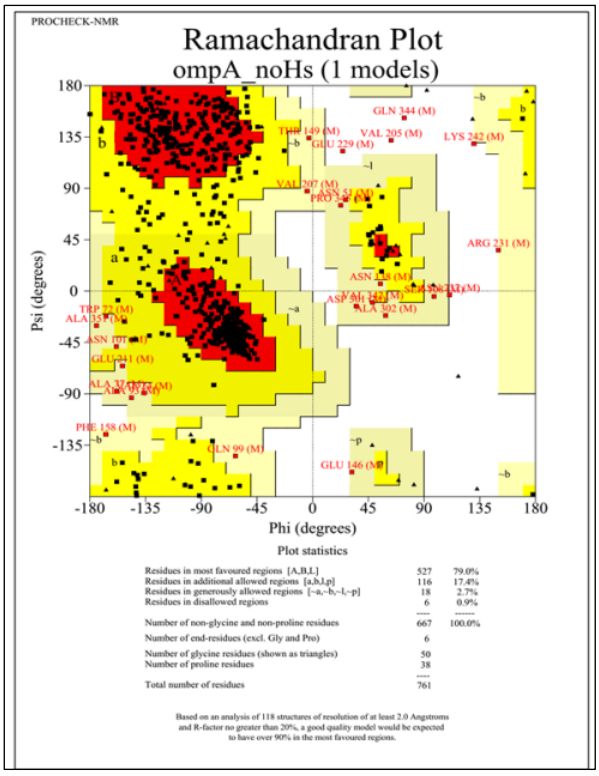

Figure 4.Ramachandran plot of a predicted good model for outer membrane protein A (*OmpA)*

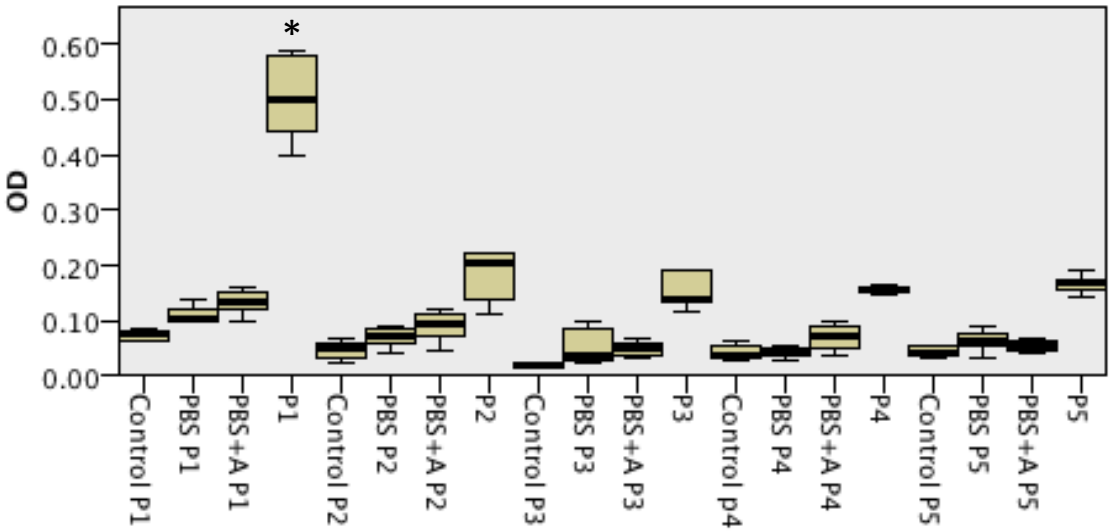

Figure 5. Mice were injected with different peptides (P1, P2, P3, P4, and P5), PBS (sham) and phosphate buffered saline (PBS) plus adjuvant (sham) separately and euthanized 14 days after the last injection. No statistically significant difference was observed between sham-injected groups and p2, p3, p4, and p5. Conversely, we observed statistically a significant difference among immunized mice with p1 and other groups ($p<0.05$).

DISCUSSION

In the present study, we showed the efficacy of OmpA antigenic epitopes as part of novel vaccine design. As reported by Thomas et al [21] peptides can stimulate immune responses and make immunization more efficacious and cost-effective than those that have been known to date. In addition, our results showed that the peptides have immunogenicity, as was shown by Martino et al[22] To date, several *A. baumannii* antigens have been evaluated as potential vaccine candidates; however, the protective capabilities of these antigens have been limited.[1] The first vaccines used killed or attenuated forms of pathogens to protect the host against subsequent infections; while these vaccines contained from tens to a few hundred proteins, only a few of these proteins induced strong immune responses. Single proteins also include many antigenic peptides, but not all of these peptides induce strong immune responses. Some peptides are antigenic determinants within larger proteins and can induce protective immune responses.[23] Effective responses against antigenic peptides should concentrate on immunogenic regions in order to avoid non-protective responses, immune evasion, and unwanted side effects. Therefore, epitope-based vaccines may be effective in the treatment and prevention of infectious diseases.[24] Structural vaccinology is a developing field currently being applied against group B streptococci,[21] pathogenic *Neisseria* species,[25] and influenza virus.[26,27] In the current study, we used bioinformatics and computational methods to predict T cell and B cell epitopes in order to accelerate the process of selection of antigens that stimulate strong immune responses. This eliminates the need for the intact protein; thereby saving time and resources.[28] No one to the best of our knowledge has studied the application of peptides of OmpA as a vaccine against *A. baumannii* infections and we showed peptide 1 (24-50 amino acids) is more immunity than others. One of the important reasons to may justify immunity this peptide is biochemical properties, so that peptide 1 is more stable with a longer half-life than other peptides. In addition, the availability of antigen epitopes is also an important application of structural prediction; hence, we predicted tertiary structure; which allowed us to select the epitopes that are exposed to the extracellular milieu and are available to antibodies. On the other hand, in order to induce both humoral and cellular immune responses, it was important to incorporate both T cell and B cell epitopes because an effective *A. baumannii* vaccine should comprise epitopes that trigger both arms of the

adaptive immune system. Finally, we mention that the limitation of this study was related to the low number of mice. We were not able to use parametric tests for data analysis, as statistical tests normally require a larger sample size to ensure a representative distribution of the population. Overall, this study resulted in the identification of one of OmpA peptides that could be used in a vaccine protecting against *A. baumanii* infection. Moreover, our study could help circumvent some of the problems associated with protein expression and purification as well as antibody cross-reactivity.

Prediction of suitable epitopes in an antigen and the use of these epitopes as a vaccine could help the prevention and treatment of various infectious diseases. We reason that one of the OmpA peptides identified in this study could provide protection against *A.baumannii* nosocomial infections. Thereby functioning as a potential vaccine candidate as a result of their ability to potentially elicit immune responses. In future studies, we plan to investigate this peptide *in vivo* immunization and challenge experiments in order to investigate passive and active immunization and we can use this peptide as a diagnostic test for *A.baumannii.*

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