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Immunogenicity of the combination of periplasmic proteins BauA, Oma87, and Bap from *Acinetobacter baumannii* in a murine sepsis model

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Abstract

Background and Objective: *Acinetobacter baumannii* is a serious challenge to the healthcare system as a multi-drug-resistant bacterium. BauA is a siderophore of *A. baumannii*. Oma87 has been stated as an immunogenic outer membrane protein per reverse vaccinology. Biofilm-associated protein (Bap) plays an indispensable role in biofilm formation by this pathogen and makes it resistant to a wide range of antibiotics and lethal conditions. The persistence and colonization of *A. baumannii* can be prevented by raising antibodies against BauA, Oma87, and Bap.

Materials and Methods: The recombinant proteins BauA, Oma87, and Bap were expressed and purified and injected subcutaneously in single and/or in combination forms to BALB/c mice and then they were challenged intraperitoneally with a lethal dose of 50% *A. baumannii* ATCC19606. The serum of combined mice was injected intravenously through the tail vein to create passive immunity in non-immune healthy mice. The mice's spleen, liver, and lung tissues were examined to check the bacterial load.

Results: Oma87 and Bap in combination with BauA in the mouse model brought about significant immunity. Antibodies produced in mice successfully detected and bound the combination of antigens in the mouse model. After immunizing and challenging mice with *Acinetobacter baumannii* ATCC19606 admixed with mucin 100%, the survival rate was monitored. Passive immunization using the sera of mice immunized against Oma87 and BauA as well as BauA and Bap yielded 85.7% survival.

Conclusion: The combination of BauA with Oma87 and Bap brought about higher protectivity against *A. baumannii* infection than their individual administration.

Keywords: *A. baumannii*, BauA, Bap, Oma87, Immunogen

1. Introduction

Acinetobacter baumannii is one of the most important nosocomial opportunistic pathogens that causes infections worldwide, especially in intensive care units (1). *A. baumannii* infections are associated with organs and body systems that have high fluid exchange rates such as the peritoneal system, urinary tract, and respiratory tract (2). In Asian countries, multidrug-resistant (MDR) *Acinetobacter baumannii* is an important threat to human health and is the major cause of hospital-acquired infections including pneumonia, sepsis, bacteremic wounds, and urinary tract infections (3). The success of *A. baumannii* can be attributed to several factors: (i) its ability to form biofilms and resist damage on abiotic surfaces (e.g. medical devices and environmental abiotic surfaces); (ii) its ability to adhere to colonization and invasion of human epithelial cells; (iii) a robust set of antibiotic resistance mechanisms that can be rapidly upregulated if necessary, and (iv) their ability to acquire foreign genetic material from the environment through horizontal gene transfer to promote survival under antibiotic and environmental pressures and receiving host selective genes. Several known pathogenic factors such as capsular polysaccharide,

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lipopolysaccharide, phospholipase, protein secretion system, two-component efflux pumps, and biofilm formation are associated with this pathogen. The new biological characteristics that are rapidly under stress conditions are formed and complicate the pathogenesis (4). *A. baumannii* has been reported to inhibit available treatment options. In this dire situation, the need for new approaches to treating infections with this pathogen is inevitable (1). Biofilms are highly structured cohesive communities in which microorganisms adhere to surfaces and are enclosed within a polymeric matrix called exopolysaccharide (EPS), also of particular importance in medicine (5-7). Biofilm production in *A. baumannii* may promote increased colonization and persistence, leading to higher rates of organ-associated infections (6). The bap gene contains 25,863 base pairs and encodes a biofilm-associated protein (Bap) with a molecular weight of about 854kDa (8). Bap promotes primary binding for cell-to-cell interactions in *A. baumannii* and other bacteria (9). The seven tandem repeats of Bap, which form the core components of the functional and conserved regions, have a strong tendency to bind to each other and form biofilms (10). Iron, as the fourth most abundant element in the earth's crust (11), is an essential micronutrient that is required for the metabolism, reproduction, respiration and survival of bacteria (12). It is widely accepted that iron plays an important role in the pathogenicity of *A. baumannii* (13). Since free iron is a micronutrient confined to the host (14-16), *A. baumannii* under these lethal conditions expresses BauA, which is an outer membrane protein and acts as a siderophore-Ferric complex receptor (17, 18). Disruption of BauA function is associated with growth inhibition under iron critical conditions (19). Outer membrane proteins (Omps) are abundantly found in the outer membranes of bacteria, often exposed through the outer polysaccharide capsule, making the Omp protein family a suitable vaccine candidate. This also applies to Oma87 (20). The β-barrel assembly machine (BAM) creates a multi-protein assembly in the outer membrane of Gram-negative bacteria, which is involved in the targeting and folding of β-barrel outer membrane proteins. A basic biological process that depends on the Bam complex in OMP assembly, which can be seen in E. coli, has been discovered in the past. Oma87 in *A. baumannii* is also one of the immunogenic proteins identified as a suitable vaccine in Pasteurella multocida (21,22). The second periplasmic BamA is a helical chain of five POTRA (polypeptide translocation) domains, the barrel of BamA together with its POTRA domains, designated P1 to P5, is essential for bacterial viability (23). Current immunization strategies target one or more Omps, and such antigens are easily prepared and do not pose a safety risk because there is no risk of the bacterial infection returning to its malignant and dangerous state and complications. They have few side effects compared to complete vaccination of attenuated or killed cells. Omps act effectively as protective antigens because they are able to stimulate the host's immune system and protect against bacterial challenges (24). Our previous works show persistence and colonization of *A. baumannii* can be prevented by raising antibodies against surface exposed virulence factors. In the present study, we aimed to evaluate the combined immunogenicity of previously explored recombinant proteins of BauA, Oma87, and Bap from A. baumannii.

2. Materials and Methods

2.1. Bacteria and culture medium

Acinetobacter baumannii bacteria (ATCC 19606) and E. coli BL21 (DE3) carrying and expressing a protein containing plasmid pET28a were used. Liquid culture medium and agar culture medium were used for bacterial growth. Autoinduction medium was also used to increase the expression of BauA and Oma87 and Bap proteins

2.2. Bacterial cultures, expression and purifications of Oma87, BauA and Bap

An autoinduction culture medium was used. For this purpose, E. coli harboring the target protein expression vector were first used and increased in the culture medium. The expression of each protein was auto induced. Autoinduction medium containing 186 ml of Zy, 4 ml of 5052, 10 ml of sterile NPS, 200 µl of MgSO4, and 100 µl of kanamycin was used. After seeding bacteria into the culture medium, it was shaken overnight at 37°C and then the bacteria were precipitated at 5000 rpm at 4°C for 20 minutes. The bacterial sediment was dissolved in Tris EDTA. The obtained bacterial suspension was sonicated at 200 w for 60 s with a 60 s cooling period between each burst and the sonicated preparation was centrifuged at 13000 × g for 20 min at 4°C. Sediment was collected from the obtained solution and the obtained bacterial debris was dissolved in TE and centrifuged. Finally, the supernatant solution and bacterial debris were dissolved in 8 M urea buffer and re-sedimented. 250 µL of supernatant was mixed with 250 µL of SDS-PAGE sample buffer and loaded on 12% SDS-PAGE gel prior to electrophoresis. The gel was immersed in Coomassie blue R-250 for 15 min at room temperature. 1.5 mL of supernatant samples that were positive for soluble protein were applied to a 2 mL Ni-NTA column. The column was washed with buffer and eluted with 500 µL of 250 mM imidazole to isolate the pure protein. Finally, the chromatography column was washed with distilled water, filled with 20% ethanol, and stored at 4°C. The Bradford colorimetric method was used to determine the protein concentration.

2.3. Study outcomes

A 12% SDS-PAGE gel was subjected to electroblotting using standard conditions and the blot was immersed in transfer buffer (150 mM glycine, 20 mM Tris-base, and 20% methanol), and incubated for 1 h at 4°C in 3% BSA blocking buffer with gentle shaking. The membrane was incubated with pooled antiserum samples at a dilution of 1:2,000 for 2 h and then incubated with a horseradish peroxidase secondary antibody at a 1:10,000 dilution with gentle shaking for 2 h at room temperature. The membrane was washed three times with phosphate buffered saline with 0.05% Tween 20 (PBST) and the immunoblot was developed using 3, 3-diaminobenzidine (Sigma).

2.4. Laboratory animals and maintenance conditions

Five groups of seven and two groups of eight 8-week-old BALB/c mice were transferred and delivered from Royan Institute (Tehran) to Shahed University animal care center under recommended conditions and regulations.

2.5. Immunization of animals

The control group was treated with sterile PBS with adjuvant. Three groups of seven mice were immunized with BauA, Bap, and Oma87 and two groups of seven mice were immunized with the combination of BauA with Oma87 and BauA with Bap in combination with complete adjuvant in the first injection and in combination with incomplete adjuvant on days 15 and 30 of injection. In each injection, 20 µg/ml protein was injected into each mouse. The next two groups of seven belong to the combined group of both proteins (BauA-Oma87) and (BauA-Bap), which in the first injection, the proteins were in combination with complete adjuvant. The next two injections were used on days 30 and 45 with incomplete adjuvant. The serum of each group of mice was maintained at -70°C.

2.6. Antibody response

The antibody titer was determined by ELISA. In brief, 5 µg/ml protein in combination with bicarbonate buffer was placed in each 100 µl well and incubated for 2 hours at 37oC. After draining the plate, the wells were washed with PBST (0.05% Tween 20) and incubated with BSA for 60 minutes. After draining the plate and washing it with PBST, serum was prepared at 1:124,000 dilutions and placed in the wells and incubated for two hours and then incubated with HRP at a dilution of 1:15,000 for one hour. After washing the plate with PBST and TMB, the staining reaction was performed in dark. The reaction was stopped with H2SO4 after 25 minutes at a concentration of 3 N. A wavelength of 450 nm was used for reading.

2.7. Determination of LD50

50% lethal dose (LD50) of *A. baumannii* ATCC 19606 was determined. Bacteria reached an OD600nm of 0.6. The bacterial suspension was sedimented and the precipitate was washed three times with PBS. The final precipitate was dispersed in PBS and serial dilutions were made. Dilution of 6.7×107 was determined as LD50 and 6.7×106 admixed with mucin were injected intraperitoneally.

2.8. Passive immunization

The sera from mice group immunized with the combined proteins of BauA and Oma87 and BauA and Bap were prepared. The sera were inactivated for half an hour at 56°C. 100 microliters of each serum were injected into each mouse via the caudal vein of non-immune healthy mice. After 3 hours, a bacterial suspension at LD50 dose admixed with mucin was injected intraperitoneally into each mouse and the survival rates of these groups of mice were monitored for 96 hours.

2.9. Bacterial load in internal organs of actively immunized mice

The 50% lethal dose of *A. baumannii* was injected intraperitoneally in combination with mucin at a ratio of 1:4. The mice were euthanized after 48 hours and the liver, spleen, and lungs of the immunized mice were removed under sterile conditions and mixed with sterile PBS at a ratio of 1:1000 (W/V). The resulting homogenate was cultured on LB agar plates and incubated overnight at 37oC. The number of colonies per gram organ enumerated.

2.10. Statistical analyses

All statistical analyses were performed using GraphPad Prism 9 software. Data were presented as mean with standard deviation. The non-parametric logarithmic rank was also used to evaluate the survival level. Antibody titers were compared using Tukey's comparison test. If the p value was less than 0.05, the differences were considered significant. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3. Results

3.1. Protein purification and Western blotting

All three proteins Oma87, BauA, and Bap were insoluble. After dialysis of the proteins in 250 mM imidazole, the protein was analyzed on the SDS-PAGE gel to ensure the correct folding of the protein (Fig. 1A). Western blotting confirmed the proteins (Fig. 1B).

3.2. Indirect ELISA

The results showed significantly elevated induction of antibodies in the mice groups that received Bap-BauA or Oma87-BauA. The group of mice that received BauA-Oma87 showed higher antibody titer than the Bap-BauA mouse group (Fig. 2A.). The serum from passively immunized mice could detect Oma87-BauA and Bap-BauA antigens (Fig. 2B). In Cross ELISA, the combined mouse sera were able to identify single-coated antigens in the ELISA plate (Fig. 3).

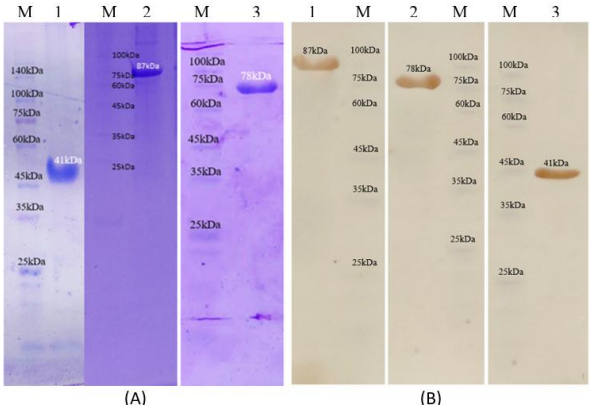


Fig. 1. (A) Protein bands on 12% SDS-PAGE gel electrophoresis
The purification of both His-tagged recombinant proteins produced in E. coli BL21 was performed by Ni-NTA affinity column, visualized by SDS-PAGE and Coomassie blue staining. From left to right, 1: Bap protein, 2: Oma87 protein, and 3: BauA protein (B) Western blot of recombinant proteins were analyzed. Column 1: Oma87, column 2: BauA, column 3: Bap, and M: molecular weight marker.

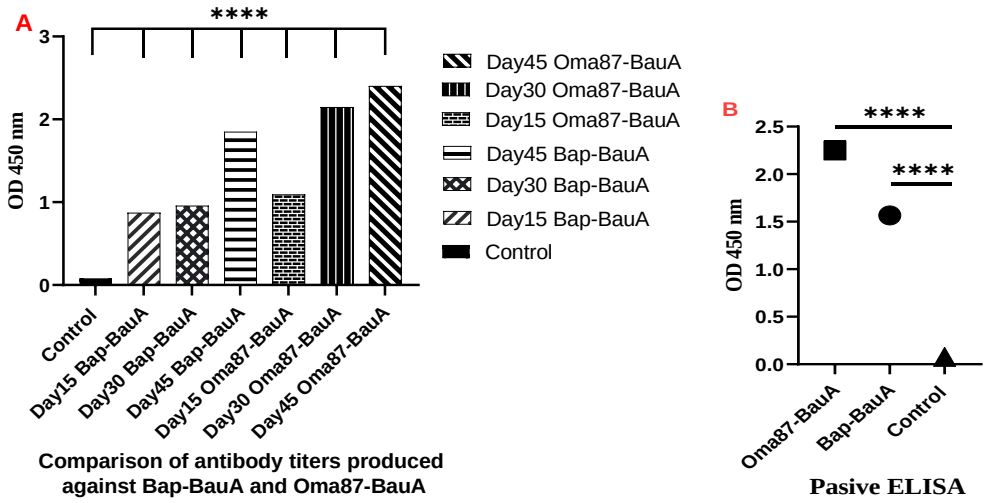


Fig. 2. Indirect ELISA of combined Oma87-BauA and Bap-BauA injected mice groups
A) The specific IgG produced in active immunization, (B) ELISA of the mice group immunized passively

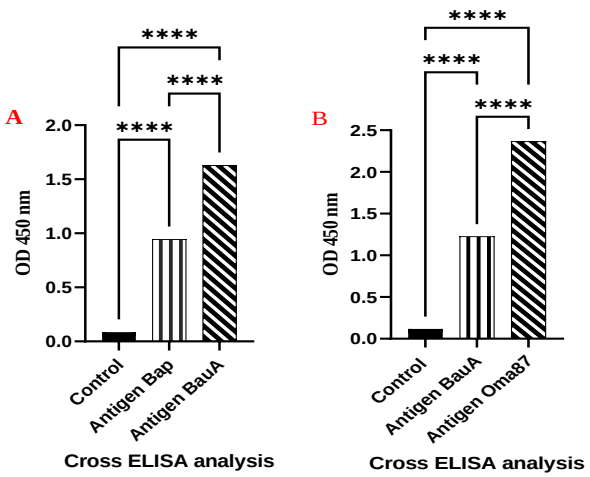


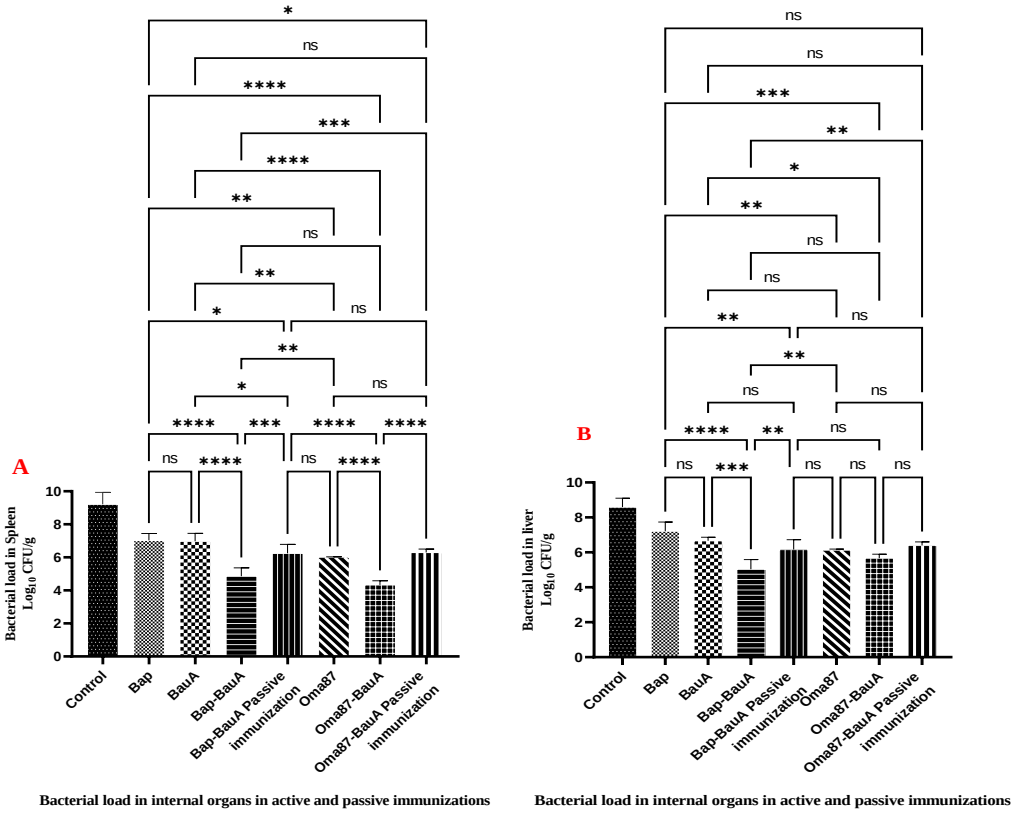
Fig. 3. (A) Cross ELISA of Bap-BauA, and (B) Oma87-BauA
P value: <0.0001 compared to the control and other single antigen administered groups

3.3. Determination of bacterial lethal dose and mice challenge

The LD and LD50 were determined as 6.7×108 CFU/ml 6.7×107 CFU/ml, respectively. The actively immunized mice monitored for 96 hours showed 100% survival. The passively immunized groups showed a survival rate of 85.7%.

3.4. Bacterial load in mouse organs

There was significant decrease in bacterial burden loaded in the spleen, liver, and lung organs of the mice immunized with Bap-BauA or Oma87-BauA. The mice group passively immunized with the sera from mice group immunized with combination of BauA and Oma87 exhibited a significant decrease in the bacterial load (Fig. 4).



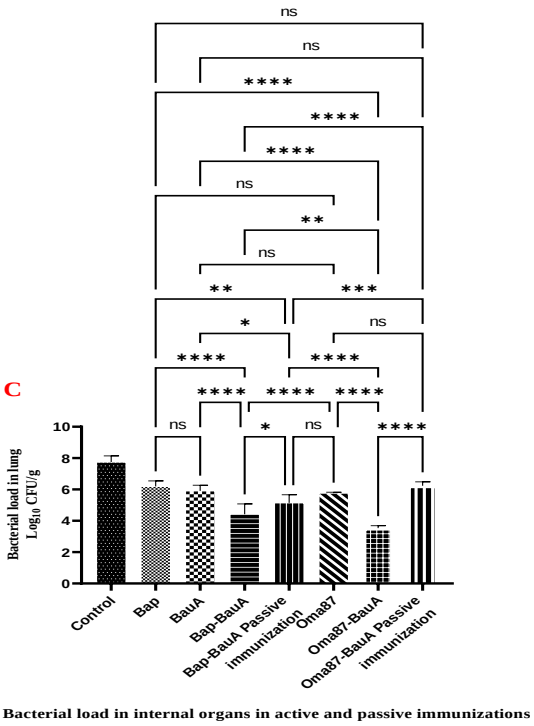


Fig. 4. The bacterial load in (A) spleen, (B) liver, and (C) lungs of passively immunized groups
P value <0.0001 compared to the control

4. Discussion

Development of vaccines against highly infectious microorganisms remains one of greatest achievements of the scientists. The primary focus is on the pathogenesis of the microorganism based on its virulence factors. Monoclonal vaccines are a new achievement for the effective treatment of *Acinetobacter baumannii* infections that are difficult to treat (25). Vaccines that have multiple antigens can achieve higher immunity than single-antigen vaccines, so they provide high relative protection (26). A combination of two or more antigens can be considered to be employed to achieve higher immunity than the single antigens. Inhibiting the mechanism of iron absorption and preventing the formation of biofilm by *A. baumannii*, which are vital for the persistence and pathogenicity of this pathogen, can be targeted (27, 28). Bap, BauA, and Oma87 are proteins with such properties. These three antigens from *A. baumannii* are very suitable for creating immunity with high protection.

Conclusion

The results of this study showed that the survival rate of the combined protein groups is as high as that of the mentioned individual proteins. In addition, the direct use of antigens in active immunity or even the use of antibodies as in passive immunity can provide better protection against *A. baumannii* infections. The results of ELISA on days 15, 30, and 45 showed induction of antibody after the first dose attaining the peak after 45 days. The selected antigens viz., Oma87, BauA, and Bap are immunogenic enough on their own to decrease bacterial load on internal body organs. Nevertheless, immunity against BauA and Oma87 as well as Bap and BauA in combination showed the highest protective effect in active immunization against *A. baumannii*. The results also indicate that the design and production of multi-antigen vaccines will induce strong and high immunity in heterologous strains of *A. baumannii*.

Ethical considerations

This research was carried out in compliance with the principles contained in the guide for the care and use of laboratory animals. The animal care protocol was approved by the Ethics Committee of Shahid University. We certify that this study was conducted in compliance with the ethical standards outlined in the 1964 Declaration of Helsinki and its subsequent amendments or similar ethical standards. code of ethics for (immunization by the combination of membrane surface proteins, Oma87 and BauA, against *Acinetobacter baumannii* infection in a mouse model) with the code IR.SHAHED.REC.1400.068 and (immunization by the combination of two selected immunogens of the surface proteins Bap and BauA) with the code IR.SHAHED.REC.1400.071.

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