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Immunogenicity of conserved cork and β-barrel domains of baumannii acinetobactin utilization protein in an animal model

Yaqub HAJIAGHATABAR SANGROODI[1], Iraj RASOOLI[2,3,*] Shahram NAZARIAN[4], Walead EBRAHIMIZADEH[5], Fatemeh SEFID[2]

[1]Department of Microbiology, School of Medicine, Shahed University, Tehran, Iran
[2]Molecular Microbiology Research Center, Shahed University, Tehran, Iran
[3]Department of Biology, College of Basic Sciences, Shahed University, Tehran, Iran
[4]Department of Biology, College of Basic Sciences, Imam Hossein University, Tehran, Iran
[5]Department of Medical Biotechnology, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran

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Background/aim: The uptake of the ferric-acinetobactin complex into the periplasmic space relies on the baumannii acinetobactin utilization (BauA) protein. BauA is composed of cork and the β-barrel domains. We constructed a recombinant protein from conserved antigenic domains of cork and the β-barrel of BauA to evaluate their immunogenic role in an animal model.

Materials and methods: The selected *bauA* domains were amplified from a purified genome of *Acinetobacter baumannii* ATCC 19606. The domains were then cloned into pET28a and the proteins expressed in *E. coli* BL21 (DE3) were purified using nickel nitrilotriacetic acid chromatography. Mice and rabbits were immunized with an intraperitoneal injection of the recombinant BauA (rBauA).

Results: The highest immune response was achieved after the third booster injection while hyperimmunity was achieved after the second booster injection in rabbits. Immunized mice challenged with live *A. baumannii* survived, whereas all unimmunized mice in the control group died after 24 h. Mice injected with 10^9 colony forming units of *A. baumannii* preincubated with pure immune rabbit sera survived. Bacterial cultures from mice spleen and liver specimens revealed the absence of bacterial growth in the immunized groups.

Conclusion: The rBauA could be used as a prophylactic agent and further tests should be carried out to see if it may be useful in a clinical setting against *A. baumannii* infections.

Key words: *Acinetobacter baumannii*, baumannii acinetobactin utilization, siderophore, vaccine

1. Introduction

Acinetobacter baumannii is a gram-negative opportunistic pathogen that causes nosocomial infections such as pneumonia, urinary tract infection, bacteremia, meningitis, and wound infection (1). The bacteria cause illness mostly in patients hospitalized in an intensive care unit (ICU) or with a compromised immune system (2). Additionally, this bacterium is emerging as multi- or pandrug-resistant, which makes treatment much more difficult (3–5). Thus, the rate of mortality is relatively high (6) and alternative therapeutic targets seem to be necessary to combat the bacterium. Iron is an essential micronutrient for all living organisms. All aerobic and anaerobic bacteria, except for *Lactobacillus* and the causative agent of Lyme disease, need iron for survival (7). Iron, functioning as a cofactor, has a significant role in the key metabolic processes and pathogenesis of bacteria (8). In mammals, free iron binds to carrier proteins such as lactoferrin and transferrin (9), and thus the iron needed for bacterial pathogenesis is restricted. This is considered to be a protective mechanism against bacterial pathogenesis (10–12). Bacteria have developed alternative mechanisms for iron uptake to overcome this limitation. One of the most important mechanisms is synthesis and secretion of siderophores. Siderophores have a low molecular weight and a high affinity to Fe(III) (13). Consequently, iron-regulated outer membrane proteins (IROMPs) are expressed on the bacterial surface to uptake the ferric-siderophore complex (14). For *A. baumannii*, iron uptake is one of the processes known to play a key role in infection and establishment in the host (15). Acinetobactin, a catecholate siderophore composed of equimolar quantities

* Correspondence: rasooli@shahed.ac.ir

of 2,3-dihydroxybenzoic acid, L-threonine, and N-hydroxy histamine, is secreted for iron chelating in *A. baumannii* (16). Uptake of the ferric-acinetobactin complex into the periplasmic space relies on the baumannii acinetobactin utilization (BauA) protein (17). BauA is the most important outer membrane protein in pathogenic *Acinetobacter* species and especially *Acinetobacter baumannii*, belonging to the TonB-dependent transporter protein family, and it is expressed under iron-limited conditions (18,19). Similar to other gram-negative bacterial siderophore-Fe receptors, BauA is composed of 2 domains, the cork and the β-barrel. The latter is composed of 22 transmembrane antiparallel β-strands. The cork is the smallest domain of the protein and is composed of 4 antiparallel hydrophobic β-strands at the N-terminal. The cork domain functions as a plug that keeps the barrel domain channel closed in the absence of the ferric-acinetobactin complex (18). This study encompasses construction of a recombinant protein from conserved antigenic domains of cork and the β-barrel to evaluate their immunogenic role in a mice model.

2. Materials and methods

2.1. Selection of antigen

The conserved antigenic regions of BauA were chosen from our earlier report (18). Epitope mapping studies resulted in the selection of the plug region and the β-barrel domain of the protein. The selected recombinant BauA used in this study was constructed from the coding regions of the cork domain to loop 9 end of the *bauA* gene of *A. baumannii* ATCC 19606.

2.2. Gene cloning, protein expression, and purification

Two specific primers (forward: 5′-GGATCCATGGATAA TTCAACAAAAACTCTA GAAC-3′, reverse: 5’-AGATC CCACTCTGCTCCAA-3′) were designed for the isolation and amplification of the selected bauA domains from the purified genome of *A. baumannii* ATCC 19606. The selected DNA segment was then cloned into the pET28a (+) (Novagen Company, USA) expression vector by *Bam*HI and *Sal*I (Fermentas, Denmark) as 5’ and 3’ restriction enzymes, respectively. The selected *bauA* gene contains an *Nde*I restriction site on nucleotide 894. The cloning process was analyzed by plasmid digestion and restriction mapping with an *Nde*I restriction enzyme. Recombinant constructs were transferred into *E. coli* BL21 (DE3). Transformed bacteria were cultured in Luria-Bertani (LB) medium containing of 70 μg/mL of kanamycin. A single bacterial colony was cultured in LB medium and incubated until the optical density at 620 nm reached 0.7. The expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was incubated at 37 °C at 250 rpm for 4 h. Cells were collected by centrifugation at 10,000 × *g* for 10 min. The pellet was resuspended in lysis buffer (100 mM H_2PO_4, 10 mM Tris base, 8 M urea, pH 8). Cells were disrupted by sonication and the supernatant was discarded after centrifugation at 12,000 × *g* at 4 °C for 20 min. Expression was analyzed by 9% SDS-PAGE. The recombinant protein BauA was purified using nickel nitrilotriacetic acid (Ni-NTA) chromatography with gradient reduction in pH. Purification was examined by 9% SDS-PAGE. Urea was removed by dialysis in phosphate-buffered saline (PBS) and reduced concentrations of urea. Protein concentration was calculated by the Bradford method (20).

2.3. Animal husbandry

BALB/c male mice, of 4–6 weeks old and weighing 16–22 g, and New Zealand White male rabbits were procured from the Razi Institute, Tehran, Iran. Mice were housed under standard, clean, and well-aerated conditions in the animal care facility at Shahed University. Research was conducted in compliance with the Animal Welfare Act and regulations related to experiments involving animals. The principles stated in the Guide for the Care and Use of Laboratory Animals were followed (21). The animal care protocol was ethically approved by Shahed University.

2.4. Animal immunization

Fifteen mice were immunized with an intraperitoneal (IP) injection of 10 μg of the recombinant BauA (rBauA) emulsified in an equal volume of complete Freund’s adjuvant. The subsequent 3 booster doses were given with 10 μg of rBauA emulsified in incomplete Freund’s adjuvant. The control mice received an equal amount of adjuvant alone. The same procedure was applied to the rabbits with 100 μg of rBauA.

2.5. Determination of antibody titers

The immune response in mice and rabbits was analyzed by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, 5 μg of rBauA was coated with 100 μL of bicarbonate/carbonate coating buffer (100 mM, pH 9.6) in a 96-well ELISA microplate. The plate was incubated at 4 °C for 16 h. Each well was washed with 200 μL of PBS-T (PBS buffer 1X + 0.05% Tween 20). Serial dilutions of sera were prepared, 100 μL of which was added to the designated wells, and the plate was incubated for 2 h at 37 °C. The wells were washed 3 times with 200 μL of PBS-T. Then 100 μL of antimouse or antirabbit antibody conjugated with horseradish peroxidase (HRP) was added to the wells treated with mouse or rabbit sera, respectively. After 1 h of incubation at 37 °C, the wells were washed as before and each well received 100 μL of TMB substrate. The immunoreaction was stopped after 15 min with the addition of 100 μL of 3 N H_2SO_4. The absorbance was read at 450 nm.

2.6. Western blotting

Western blotting with anti-His-tag antibodies (QIAGEN, USA) was carried out to confirm protein expression.

The rBauA was transferred from 12% SDS-PAGE gel to nitrocellulose paper using a Bio-Rad Mini-Protean Tetra System run for 60 min at 75 V. The nitrocellulose paper was stained with Ponceau S and destained with PBS (pH 7.2). Nitrocellulose paper was incubated with blocking buffer (5% skimmed milk in PBS-T [PBS + 0/05% Tween 20]) overnight at 4 °C. After washing 4 times with washing buffer (PBS-T) on the shaker for 5–10 min each time, anti-His-tag antibodies were added to a final concentration of 1/1000 and incubated for 1 h in 37 °C. Washing was repeated as described above. The substrate buffer (50 mM Tris-HCl (pH 7.2), 0.6 mg/mL DAB, and 1 µL/mL H_2O_2) was added and the reaction was stopped with PBS.

2.7. Chrome azurol S assay

The bacterial ability to grow in the presence of 100 µM iron chelator 2,2′-dipyridyl (DIP) (Sigma-Aldrich, Munich, Germany) was determined by using tryptone soy broth supplemented with 1% NaCl as a basal medium. This compound had no effect on the pH of the medium or on the growth rate of the bacterium (22). To evaluate the capacity of siderophore production by *A. baumannii* ATCC 19606, a universal chemical assay on chrome azurol S (CAS) agar plates was used (23). The bacterium was cultured on tryptone soy agar medium supplemented with 1% NaCl for 48 h at 37 °C. The assay was performed by spotting 10 µL of bacterial culture onto CAS agar. The siderophores produced by *A. baumannii* ATCC 19606 on plates were detected by the appearance of an orange halo around microbial colonies after 72 h of incubation up to 7 days postinoculation.

2.8. Determination of lethal dose (LD_{50})

The lethal dose (LD_{50}) was calculated in mice by IP injection of *A. baumannii* at doses ranging from 10^4 to 10^9 CFU into 6 groups of 5 BALB/c mice. For challenge assay, the immunized mice were randomly divided into 3 groups of 5 animals. Mice in groups 1, 2, and 3 were challenged with IP injection of 10^8, 10^9, and 10^{10} CFU of *A. baumannii* ATCC 19606, respectively.

2.9. Neutralization test

For neutralization assay (24), 4 groups of healthy mice were taken under study. Groups 1 and 2 were further subdivided into 5 groups of 5 animals. *A. baumannii* at 10^9 and 10^{10} CFU concentrations was mixed with 200 µL of immune rabbit serum at pure, 1/250, 1/500, 1/1000, and 1/5000 ratios and incubated for 30 min at 37 °C. The mixtures were then injected intraperitoneally to the mice groups. To analyze the specificity of immune response against BauA protein, 10^5 CFU of *Staphylococcus aureus* with LD_{50} of 10^4 was mixed with 200 µL of pure immune rabbit serum and injected intraperitoneally into the mice in group 3. Group 4 received 200 µL of rabbit serum only, to serve as the control. The animals were monitored for mortality for 1 week.

2.10. Monitoring establishment of *A. baumannii* infection

The immunized and unimmunized mice inoculated intraperitoneally with 10^9 and 10^{10} CFU of live *A. baumannii* were sacrificed and the liver and spleen were removed aseptically to test for the presence of *A. baumannii*. Each organ was homogenized separately and 0.1 g of each homogenate was suspended in 1 mL of PBS. Then 100 µL of each suspension was cultured in LB agar. The bacterial colonies were counted (25) and suspensions were prepared to verify the presence of *A. baumannii* through routine laboratory methods. Livers and spleens from healthy animals served as the control.

2.11. Statistical analysis

The data are expressed as mean ± standard deviation (SD). P-values were calculated by Student's t-test to determine the significance of differences in the experimental groups. $P < 0.05$ was considered significant.

3. Results

3.1. Cloning

PCR amplification of the *rBauA* gene resulted in a 1800-bp fragment. The fragment was purified and cloned into a pET28a (+) vector. Cloning was confirmed by digestion with the cloning restriction enzymes and restriction mapping with *Nde*I. There is an *Nde*I restriction site both on the pET28a (+) vector at nucleotide 239 and on *bauA* at nucleotide 894. Restriction mapping resulted in a 934-bp fragment, confirming the cloning of the selected *bauA* gene.

3.2. Protein expression and purification

Induction with 1 mM IPTG brought about a high expression of rBauA. Inclusion bodies were dissolved in a lysis buffer containing 8 M urea. SDS-PAGE analysis revealed a thick protein band of 68 kDa. Ni-NTA chromatography purification resulted in a single band of 68 kDa designated as rBauA (Figure 1). The expression was confirmed with western blotting using HRP-conjugated anti-His antibodies (Figure 2).

3.3. Animal immunization, challenge, and neutralization test

The highest immune response in BALB/c mice was achieved after the third booster injection while hyperimmunity was achieved after the second booster injection in rabbits (Figure 3). The difference in antibody titer between the first and second doses of immunization was statistically significant ($P < 0.003$). The rise in antibody titer after the third immunization was also significant ($P < 0.004$), while there was no statistically significant rise after third dose.

3.4. Determination of lethal dose (LD_{50})

The LD_{50} for *A. baumannii* was determined as 10^8 CFU for BALB/c mice. Immunized mice were challenged with live

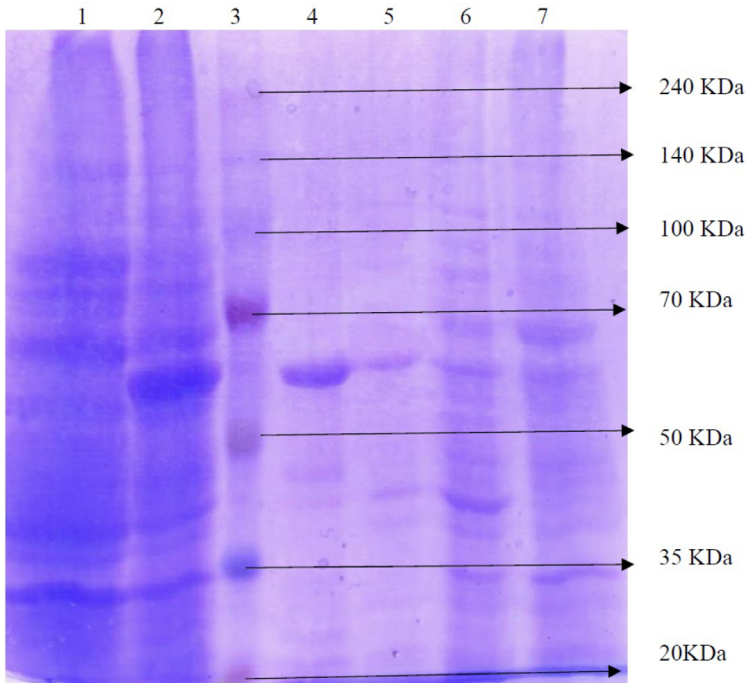

Figure 1. SDS-PAGE analysis of recombinant BauA protein. Lane 1: Uninduced control, Lane 2: expression of rBauA protein induced with 1 mM IPTG, Lane 3: standard protein molecular weight marker, Lane 4: purification of rBauA protein with QIAGEN buffer E, Lanes 5 and 6: column effluent after washing with QIAGEN buffers D and C respectively, Lane 7: column effluent after loading induced lysed cells.

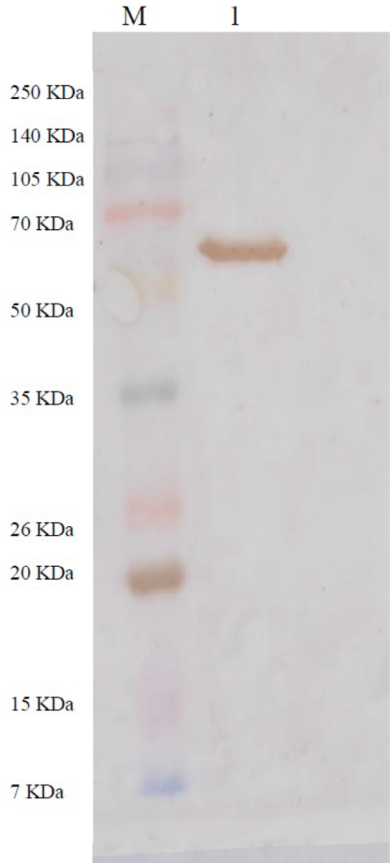

Figure 2. Western blot analysis of rBauA protein. Lane 1: 68-kDa band refers to the purified rBauA protein; Lane M: Protein molecular weight marker.

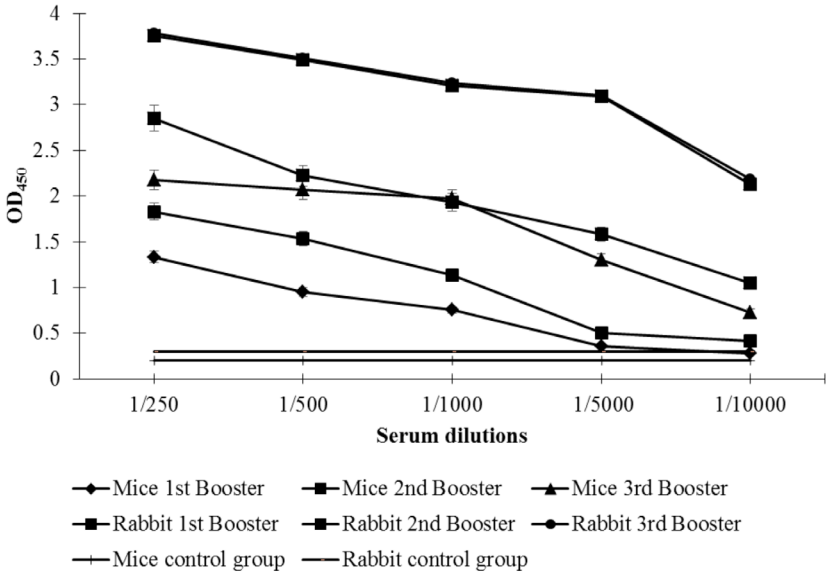

Figure 3. Immunization of mice and rabbit with rBauA.

A. baumannii. Mice in groups 1, 2, and 3 survived, whereas all unimmunized mice in the control group died after 24 h.

3.5. Neutralization test

Mice injected with 10^9 CFU of *A. baumannii* preincubated with pure immune rabbit sera survived, while all higher sera dilutions did not impart protection.

3.6. Monitoring establishment of *A. baumannii* infection

Bacterial cultures from mice spleen and liver specimens revealed an absence of bacterial growth in the immunized groups. In the unimmunized mice, 6×10^4/g and 4×10^4/g bacteria were detected in the spleen and liver, respectively.

4. Discussion

A. baumannii is one of the fastest growing nosocomial infection causes worldwide. Generally, in addition to elderly and premature infants, the population under risk consists of immune-deficient people, patients hospitalized in an ICU, and patients who underwent surgical procedure or trauma (26). *A. baumannii* is a known cause of hospital-acquired infection and the spread of multidrug-resistant strains of the bacteria in hospitals leads to complexity in antibiotic mediated treatment (4). Thus, research toward discovery of new therapeutic methods that could assist or replace antibiotic treatment is imperative. Comparison between *A. baumannii* (susceptible and multidrug-resistant) and environmental *Acinetobacter baylyi* revealed that multiple factors such as pilus biogenesis, metabolism, quorum sensing, secretive system type 4, and iron acquisition are among the key virulence factors of *A. baumannii* (15). There has been increasing success in the antibody-mediated treatment of various bacterial infections (27–29). Recently, researchers have successfully used the siderophore receptor as one of the most conserved interspecies antigens to produce vaccines against meningococci, *E. coli* O157, and avian salmonella infections (30–32). In the case of *A. baumannii*, other surface antigens, such as biofilm associated protein, have been evaluated as potential vaccine candidates (33). The protective efficiency of *A. baumannii* siderophore receptor protein (BauA), a conserved antigen among the species that is a key protein in the iron intake process, against bacterial infection was evaluated in an animal model (17). Bioinformatics studies of our earlier reports showed that this protein exists in all pathogenic *A. baumannii* strains (18). The CAS assay results showed that this bacterium absorbs iron from the environment and in iron-deficient media secretes siderophores for iron absorptions. Similar studies in BALB/c mice indicates that monoclonal antibodies against IROMPs of *A. baumannii* have in vitro bactericidal and opsonization effects and can inhibit iron absorption and siderophore function in the bacterium (34). In this study, instead of cloning all the proteins expressed in the iron-deficient state, a recombinant version of BauA protein was used. Moreover, with the assistance of bioinformatics studies, conserved antigenic regions were selected to ensure a protective immune response and less side-reaction with the use of smaller proteins (18). The ELISA results showed maximum antibody titer after the third booster injection in mice. Neutralization assay using rabbit serum against a bacterial load of 10^9 showed successful inhibition of the infection with only equal

volumes of pure serum. This probably shows the important role of the opsonization effect of these antibodies in the eradication of infection, because all immunized mice could tolerate higher doses of bacterial challenge with signs of neither infection nor colonization in internal organs. The immunized mice could tolerate 100 times the normal LD_{50} dose. The spleen and liver microbiology of the immunized mice revealed an absence of *A. baumannii*, confirming the efficacy of the antibodies produced against rBauA. rBauA was able to create rapid induction of humoral immunity within 10 days after the first injection dose in rabbits, which is an important factor in immunogenicity. The rapid growth of multidrug-resistant strains of *A. baumannii* require significant attention to be given to nonantibiotic methods of treatment. As shown in our experiments, the mice injected with *A. baumannii* preincubated with sera from rabbits immunized with rBauA survived. Production of vaccines that could effectively prevent bacterial colonization could significantly aid diagnostic and therapeutic aspects of nosocomial infections caused by this bacterium. The results support the efficacy of rBauA as a prophylactic agent against *A. baumannii* infections. The mice model presents an advantage in testing immunization with recombinant proteins, especially in *A. baumannii* infections where the animals are easily infected with the bacterium. The lack of clinical experiments on humans may be regarded as a shortcoming of the study.

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