Original Article

Confrontation with Acinetobacter Baumannii by Exploiting its Biofilm: An Insight to Vaccine Development

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Abstract

Objective: Acinetobacter baumannii is an opportunistic pathogen that causes serious infections in humans by colonization of medical devices. The capacity of this pathogen to persist in hospital settings could be due to its ability to form biofilms. In the present study we evaluated the effect of antibodies against one of surface components of A. baumannii on in vitro biofilm formation.

Materials and Methods: The 1113 bp fragment of Bap (biofilm associate protein)gene from A. baumannii genome was amplified and cloned. The recombinant protein was expressed and purified and used to raise antibodies in mice. Antibody titer was evaluated by ELISA. In vitro biofilm inhibition was evaluated using the mice sera.

Results: Injection of Bap subunit resulted in high titers of antibody. Immunized mice serum had significant ($P< 0.001$) inhibitory effect on biofilm formation. In vitro inhibition of biofilm formation by A. baumannii was studied at 1:50 concentrations of immunized and unimmunized mice sera. Immunized mice serum had significant ($P <0.001$) inhibitory effect on biofilm formation

Conclusion: Adsorption of antibodies to Bap might interfere with bacterial adhesion to the surface. These antibodies were found to be potent biofilm inhibitors.

Keywords: Acinetobacter baumannii; Biofilm associated protein (Bap); Biofilm; antibody

Introduction

Acinetobacter baumannii spp. are highly troublesome nosocomial pathogens and cause severe infections specially in intensive care units. The most concerning issue is the immense ability of the organism to acquire antimicrobial resistance.(1, 2)

One of the most important mechanisms underlying the clinical success of A. baumannii is biofilm formation on medical devices.(3,4) Biofilms are highly structured sessile communities in which microorganisms adhere to the surfaces and are encapsulated within exopolysaccharides. (5,6) Biofilms can tolerate up to 1000 times more antibiotics and disinfectants than planktonic counterparts. (6,7) Several studies have documented the ability of A. baumannii to form biofilms on glass and plastic surfaces as well as epithelial cells. (8, 9)

The precise mechanisms involved in the establishment and progression of A. baumannii infection are unclear. The organism is not known to produce either diffusible toxins or cytolysins, and a few virulence factors have been identified.(10) Biofilm-dependent production of poly-β-(1-6)-N-acetylglucosamine (PNAG) which is a known virulence factor in various PNAG-producing bacteria, could be an important virulence factor for this emerging pathogen. (11) Type I pilli are responsible for biofilm formation of A. baumannii on abiotic surfaces. (4,12) A surface protein commonly known as biofilm associate protein (Bap) has been identified in a bloodstream isolate of A. baumannii. BapA. baumannii is involved in intercellular adhesion within the mature biofilm.(13) Bap-related proteins are present on the bacterial surface and confer upon bacteria the capacity to form a biofilm, show a high molecular weight, contain a core domain of tandem repeats, and play a relevant role in bacterial infectious processes. (13-16) Certain Bap subunits have recently been

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introduced as potential immunogenic molecules via an immunoinformatic study.(17) It seems necessary to study biofilm inhibition and dispersion as biofilms are involved in 80% of human bacterial infections.(18) In the present study we use a 371 amino acid subunit of our in silico findings to analyze the in vitro biofilm inhibition potentials of antibody against Bap subunit.

Materials and Methods

2.1. Animals

BALB/c mice, 4–6 weeks old (16–22 g), were procured from the Razi Institute, Tehran, Iran. The mice were housed in clean standard 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.

2.2. Construction of plasmid

PCR (polymerase chain reaction) was performed using primers developed against sequence of Bap gene in A. baumannii. The forward primer, was (5′-TTCTAGAATTCATGGCAAATACAGTGGTCACTGTTGTA-3′), corresponding to amino acid positions 706 to 714 of the mature 854-kDa protein (Bap) of A. baumannii. The reverse primer, was (5′-TCTATAAGCTTTTATGTCGGATCGTTCACTGTACCA-3′), corresponding to amino acid positions 1054 to 1061. Forward and reverse primers had 5′end EcoRI and HindIII restriction sites respectively (underlined) for insertion into an expression vector. PCR amplification was performed using 1X PCR buffer [50mM Tris/HCl, 10 mM KCl, 5mM(NH4)2 SO4, pH 8.3], 0.2 µl of 5U/µl of Taq DNA polymerase, 1 µl of 10 mM dNTPs mixture, 2.5 µl (50ng/µl) DNA, 1 µl of each primer (10pM), 3mM MgCl2 in a final volume of 25 µl on a thermal cycler (TECHNE Gradient, Staffordshire, UK). The amplified fragments were resolved on a 1% agarose gel and visualized with EtBr. To confirm authenticity of fragments, restriction mapping was conducted on PCR product with BclI restriction enzyme. The PCR product was digested with EcoRI and HindIII and inserted into the pET28a(+) vector at the corresponding sites. The new construct was named pET28a(+)–AbBap. The resulting plasmids were transformed into Escherichia coli BL21(DE3). The transformants were grown in LB medium containing 50 µg/ml kanamycin. Surviving colonies were picked up for further analyses.

2.3. Expression and Purification of Recombinant Protein

Escherichia coli BL21(DE3) harboring the pET28a(+)–AbBap expression constructs were grown in Luria–Bertani medium supplemented with 50 µg/ml kanamycin at 37°C with shaking (220 rpm) to an OD600 of 0.6, and then induced with 1 mM IPTG. After 4 h of growth at 37°C, cells were collected by centrifugation at 5000 rpm, and stored at -80°C until further use. The pellets were resuspended in phosphate-buffered saline (PBS, pH= 7.4) and boiled at 100°C for 5 min with the sample buffer solution. The recombinant Bap subunit was purified by Ni–NTA affinity under denaturating condition as per Qiagen instructions. The cell pellet was thawed at 4°C and resuspended in buffer B (denaturating lysis/binding buffer). The lysate was then sonicated (6 times, 10 s at 200Wwith a 10 s cooling period between each burst) using a sonicator equipped with a microtip. The lysate was then centrifuged at 14000 rpm for 25 min at 4°C to pellet the cellular debris. The supernatant was applied to a Ni–NTA agarose column. The BapA protein was eluted by a stepwise procedure, using buffer C (denaturating wash buffer, pH=6.3), buffer D (denaturating elution buffer, pH=5.9) and buffer E (denaturating elution buffer, pH=4.5), all containing 8M urea. The refolding of the purified protein was achieved by sequential dialysis against PBS containing 6, 4 and 2 M urea respectively and finally in PBS (pH 7.4) buffer and stored at -80°C until use. Concentration of purified protein was determined according to Lowry et al.(19) method with bovine serum albumin (BSA) as standard.

2.4. SDS polyacrylamide gel electrophoresis

SDS-PAGE was performed by the modified Laemmli method20 with an acrylamide: bisacrylamide ratio of 30:0.8 and an acrylamide concentration of 10% w/v for the running gel and 5% w/v for the stacking gel. Molecular weights were determined using standard proteins molecular weight markers (Fermentase SM671).

2.5. Immunization of mice

Twenty mice received three vaccinations of 10µg of the recombinant Bap protein at 2 week intervals. The initial vaccinations were emulsified with complete Freund's adjuvant (Sigma), and the next two with incomplete Freund's adjuvant (Sigma). Blood samples were collected 10 days post-injection through infra-orbital plexus. An additional BALB/c mice that received PBS only, served as a control group. (20)

2.6. Analysis of antibody response

Recombinant Bap subunit was first diluted with coating solution to an optimal concentration (20µg/ml) in order to coat a 96-well plate with. The resulting solution was then added into each well (100 µl per well) and incubated for 12–18 h at 4ºC. To block the unoccupied sites, wells were washed once with PBS plus 0.05% Tween 20 (PBS-T), and then incubated with 100 µl of PBS-T plus 5% skimmed milk for 1 h at 37ºC. After washing the plates 3 times with PBS-T (100 µl per well), serial dilutions of each serum ranging from 1:800 to 1:102400 were added to the wells in triplicate and incubated at 37ºC for 1 h. Plates were washed 3 times, again as described above. 100 µl per well of Horseradish peroxidase-conjugated (HRP-conjugated) secondary antibody (diluted 1:1,000 in blocking buffer) was added and the plates incubated for 1 h. Plates were then washed 3 times with PBS-T and were then incubated with 100 µl per well of 3,3′,5,5′-tetramethylbenzidine

solution (TMB) (Sigma) as substrate until a desired absorbance was reached. The reactions were stopped by the addition of 2M H2SO4. The optical density of the samples was measured at 450 nm using an ELISA plate reader (PERLONG DNM-9602G).

2.7. Quantification of Biofilm Formation

Biomass of A. baumannii biofilms was quantified by a crystal violet (CV) staining assay. (21) Briefly, bacteria were grown in 14-ml polystyrene culture tubes containing 2 ml of Lauria-Bertani medium. Five identically prepared tubes were used for adding each immunized and unimmunized mice sera and control tubes (no serum was added to culture medium) all these tubes named as B tubes. Also five tubes were used to measure growth in suspended culture (G tubes), and another five tubes served as controls for abiotic factors (NC tubes). Inocula were grown to the late log phase. Subsequently, the B and G tubes received 20 µl of inoculum, and all tubes (B, G and NC tubes) were incubated in 37 ºC an orbital cabinet shaker for 17 ± 1 h. Following incubation, cells in the G tubes were mixed well by gentle vortexing, and the optical densities of the cultures at 600 nm (OD600) were measured. The B and NC tubes each received 125 µl of a 0.3% solution of crystal violet (CV). After 15 min, the suspended culture was poured out, and the tubes were rinsed well with distilled deionized water (six rinses, approximately 4 ml per rinse). Any remaining CV was dissolved in 2 ml of an ethanolacetone (80:20) solution, and the absorbance at 570 nm of each resultant solution was measured spectrophotometrically. Biofilm accumulation was normalized with respect to growth, which yielded the specific biofilm formation (SBF). SBF was determined by using the following formula: SBF = (B - NC)/G, where B is the CV retained in the B tubes, NC is the amount of CV that adhered to the polystyrene tubes because of abiotic factors and G is the optical density of cells grown in suspended culture. (21)

2.8. Statistical analysis

All data are expressed as mean ± standard deviation (SD). One-way ANOVA with post hoc Scheffé tests determined statistical significance of ELISA and inhibitory property of antibodies on biofilm formation. SPSS 16.0 was used for data analysis. P values of <0.05 were considered as significant.

Results

3.1. PCR amplification of the Bap gene

The size of PCR amplicon of the fragment encoding one conserved region of Bap was 1,113 bp. The fragment of appropriate size was observed on agarose gel (**Fig. 1A**). The expected fragments of digested fragments also appeared (Fig. 1B).

Fig. 1

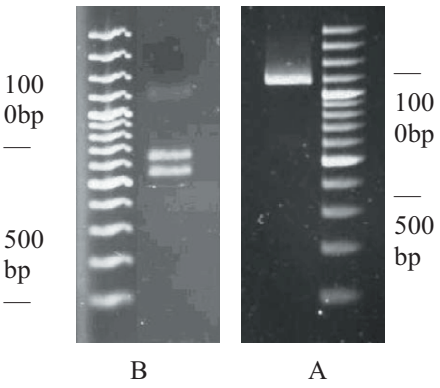

Figure 1. PCR product (A). Restriction pattern digested with *BclI* restriction enzyme, two fragments of 598 and 515 bp are visible (B). Marker: 100 bp Plus DNA ladder (Fermentas).

3.2. Expression and Purification of Bap subunit

The N-terminal hexa-His tagged protein was expressed in E. coli BL21 (DE3) and purified by immobilized metal affinity chromatography (IMAC). Average yield of purified Bap subunit was around 0.46 mg/ml of elution buffer. SDS-PAGE with Coomassie staining confirmed high protein purity. The band corresponding to desired molecular mass of approximately 41 KDa did not appear as expected (**Fig. 2**).

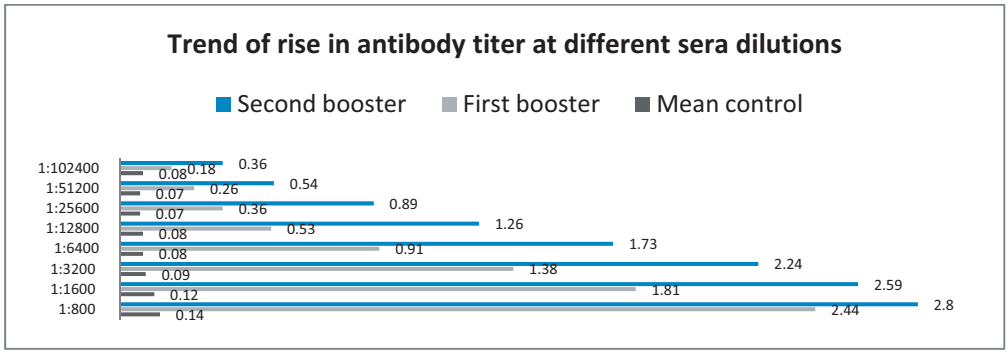

Figure 2. SDS–PAGE of recombinant Bap subunit produced in *E. coli*.

M, molecular mass standards; Lanes F1 and F2 First and second unbound protein flows; Lanes C1, C2 columns washed with washing buffer; Lanes D, E1 and E2, Column washed with elution buffers. Single band Bap subunit appeared in lane E2 but at unexpected region (see the text).

The electrophoretic mobility of the recombinant protein roughly corresponds to its molecular mass, while migration of this protein would be in accordance with the expected molecular mass of protein. This protein displays an anomalous electrophoretic behavior on standard SDS-PAGE, revealing that the protein did not bind SDS under the SDS-PAGE conditions.(22)

3.2. Antibody response

The antibody titer increased significantly (P< 0.001) after the second booster, whereas animals received adjuvant, as control had no Bap-specific antibodies in serum (**Fig. 3**).

Fig. 3

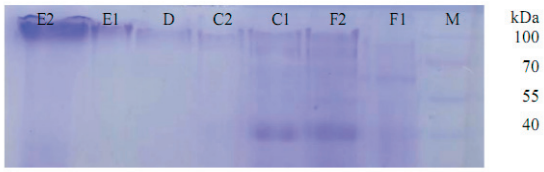

Figure 3. Indirect ELISA of serum with Bap subunit protein. Antibody titers increased significantly (*P*<0.001) in the second booster.

3.2. In vitro biofilm assay

In vitro inhibition of biofilm formation by A. baumannii was studied at 1:50 concentrations of immunized and unimmunized mice sera. Immunized mice serum had significant (P <0.001) inhibitory effect on biofilm formation (**Fig. 4**).

Fig4

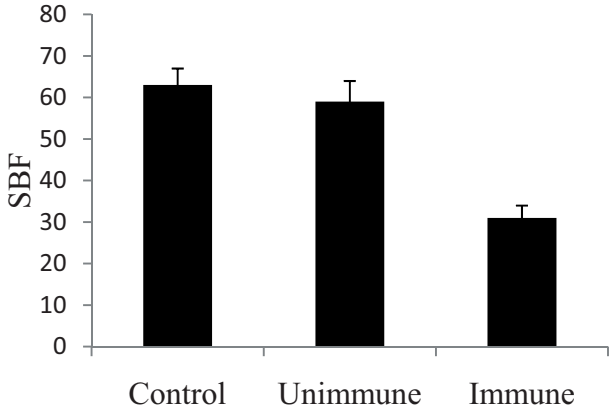

Figure 4. In vitro biofilm formation by *A. baumannii* exposed to immune and unimmune sera. The biofilm volume difference between immunized and control groups is highly significant (*P*< 0.0001). The significance of *P* < 0.001 still persists between immunized and unimmunized groups.

Discussion

Biofilms are successful survival strategy of bacteria for colonization in natural environments and several factors are involved in cellular adherence to various surfaces.(6, 23)The majority of pathogenic bacteria tend to adhere to surfaces and form biofilms. (18) A. baumannii epitomizes this trend and acquires antimicrobial resistance.(24) Hence, new strategies are needed to target biofim phenotype of this pathogen. Several attempts have been made to target this special phenotype to prevent organism from establishing biofilm. Lytic phages could provide a novel strategy for the control of biofilms on indwelling medical devices. (25) Antibiofilm activity of Oroidins, a small library of second generation of marine natural products incorporating a 2-aminoimidazole (2-AI) motif, have served as a unique scaffold for developing molecules that inhibit and disperse A. baumannii biofilm. (26) It was found that polymeric composite containing a 2-aminoimidazole derivative was resistant to biofilm colonization by A. baumannii. (27) The inhibitory effect of plant secondary metabolites against A. baumannii biofilm was also assessed, (28) anti-microbial peptides are found to be potent biofilm inhibitors as well. (29) Recent studies showed that pooled human immunoglobulins reduce bacterial adhesion on biomaterials. (30) BapA. baumannii was introduced for the first time by Loehfelm et al. 13 Brossard et al. demonstrated that the BapA. baumannii is necessary for maturation of biofilm on medically relevant surfaces, including polypropylene, polystyrene, and titanium. (31) Also, presence of Bap on the cell surface has several advantages for pathogen including adherence to eukaryotic cells which is an important step in bacterial colonization resulting in infection of the host and persistence on abiotic surfaces, although Bap is not involved in internalization of the bacterium in both normal human bronchial epithelial cells and normal human neonatal keratinocytes. (31) Furthermore, it has been shown that the presence of Bap increases the bacterial cell surface hydrophobicity. (31) Recently Bap has been utilized as a target to develop a novel vaccine that can abolish biofilm development. (32) In the present study it was found that Bap was highly immunogenic and resulted in high antibody titers (Fig. 3). Our results demonstrate that it is possible to prevent the bacterium from abiotic surface adherence with antibodies against Bap subunit. Hence, it may be proposed that antibodies against this subunit interfere with adhesion and accumulation of A. baumannii holding it in a planktonic phase and may depriving bacterium of virulence. Bap involves in early development and maturation of biofilm architecture. (13) Since steps of biofilm formation occur particularly after exopolysaccharide secretion, 6 possibility of surrounding by PNAG of Bap surface epitopes is not ruled out. Staphylococcus epidermidis reported to possess PNAG, (33) the biofilm extra matrix in this case, did not pose an overall diffusion barrier to the antibody. (34) This supports

the notion that antibodies are able to penetrate A. baumannii biofilms. Our results suggest that access to the bacterial cell surface through PNAG by antibody is sufficient to impart in vitro biofilm inhibition (Fig. 4). Reaction of antibodies against Bap with several strains of A. baumannii was previously analyzed suggesting that not only immunodominant regions of Bap in A. baumannii strains are conserved but also have the same epitope presenting pattern in different strains. Immunodominant region of Bap possesses target sites for a protective humoral immune response to A. baumannii. This seems to be a conserved region erecting efficacy of Bap as an appropriate vaccine candidate. (32) Specific and nonspecific adsorption of antibodies to Bap might inhibit adhesion and play a critical role in the prevention of development and maturation of biofilm (Fig. 4). This inhibition could simply be explained by conformational changes in native Bap while there is sufficient evidence indicating that antigens and antibodies can significantly change their molecular conformation upon binding. (30,35,36) Therefore, the strategy employed in this study confirmed that the selected subunit protein is produced in the biofilm and is exposed on the cellular envelope. The advantage of the protein exposure and its immunogenicity can be exploited in development of antibodies that might interfere with bacterial adhesion to the surfaces.

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