



A conserved region from biofilm associated protein as a biomarker for detection of *Acinetobacter baumannii*



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ARTICLE INFO

Article history:

Received 27 August 2014

Received in revised form

1 November 2014

Accepted 5 November 2014

Available online 6 November 2014

Keywords:

A. baumannii

Bap

Diagnosis

Infection

Biomarker

ABSTRACT

Acinetobacter baumannii is the leading cause of nosocomial infection within the family *Moraxellaceae*. Due to multiple antibiotic resistances of the bacteria, the treatment is very difficult, hence specific and economical test for early diagnosis of infection is needed. Development of such a test requires targeting specific cell surface antigens. Bacterial ability of biofilm formation grants major contribution in anti-microbial resistance and other environmental stresses such as nutrient limitation and dehydration. Biofilm associated protein (Bap), a specific cell surface protein, is directly involved in *A. baumannii* biofilm formation. The goal of this study is diagnosis of *A. baumannii* infection exploiting specific target from Bap. A selected subunit of Bap was cloned, expressed and purified. Mice were divided into three groups. Group one was immunized with recombinant Bap subunit, mice in group two were infected with *A. baumannii* (positive control) and mice in groups three served as negative control. Immunization with Bap subunit resulted in high antibody titers. Animals in control group that received same amount of adjuvant and PBS showed no Bap-specific antibodies. Sensitivity and specificity of the antibodies raised were determined by ELISA and Western blotting. Recombinant Bap subunit was tested by ELISA using sera obtained from *A. baumannii* infected patients and healthy individuals. This conserved and immunodominant region of Bap could serve as an appropriate target for diagnosis *A. baumannii* infection.

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1. Introduction

Acinetobacter baumannii is an aerobic, non-fermentative gram-negative rod shaped bacterium within the family *Moraxellaceae* [1]. The increasing number of infections caused by this pathogen and the prevalence of infections caused by antibiotic-resistance strains warrants the development of novel diagnostic tests, making this topic of interest. It causes nosocomial infections associated with morbidity and mortality [2] such as pneumonia, blood-stream and wound infections [3], secondary meningitis, urinary tract and burn infections [4]. Its resistance to many antimicrobial agents renders the treatment very difficult [5]. Its adherence to and persistence on abiotic and biotic surfaces as biofilm is the core pathogenicity factor of the bacteria [6]. A 25,863 bp gene encodes Biofilm-associated protein (Bap) of 854 kDa. Nucleotide sequence of this surface

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protein reveals a repetitive structure [7]. This protein contributes in bacterial stability at various environmental conditions [5]. Most strains of the bacteria can easily acquire various antibiotics resistance through different mechanisms, therefore the treatment could be a serious challenge [8]. Presence of this organism is still detected using routine time consuming culture methods such as catalase, oxidase or urease and its identification is based on biochemical assays. Delay in detection of bacteria is problematic in disease control and may result in severe complications in patient care and disease management. Therefore, a specific, fast and economical test is needed for the rapid detection of *A. baumannii* infections [9]. It is imperative to explore bacterial targets in order to develop novel approaches for diagnosis of *A. baumannii* [10]. Biofilm associated protein expression is common in clinical isolates of *A. baumannii* [7]. Outer membrane proteins (OMPs) can be used as biomarkers in the development of fast, sensitive and specific diagnostic tests for detection of *A. baumannii*. These tests are based on detection of specific immunoglobulins in patient sera [9]. Rahbar et al. [5]. reported potential antigenic structures in Bap subunit possessing various antigenic determinants that elicit antibody formation.

Hence this subunit could be used as a suitable agent for antibody–antigen based diagnostic test. Such diagnostic test helps clinicians diagnose the disease quickly and take precise initiatives to the treatment of the infection. The present study was designed to develop a method for fast and sensitive diagnosis of *A. baumannii* infections using targets presented on bacterial cell surface.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. baumannii Kh0060 (nosocomial biofilm forming strain confirmed by biochemical tests) was isolated from patients in Khatamolanbia hospital (Tehran). *Escherichia coli* BL21 (DE3) was used for protein expression. Transformants were grown on Luria–Bertani (LB) medium containing 80 µg ampicillin ml⁻¹. *Salmonella enteritidis*, biofilm forming *Staphylococcus aureus* ATCC 6538 reference strain, *E. coli* BL21 (DE3) and *A. baumannii* strains Kh0060 and ATCC 19606 were grown in LB broth.

2.2. Gene amplification and plasmid construction

A. baumannii strain Kh0060 was used for genome purification. Polymerase Chain Reaction (PCR) was performed using Bap gene specific primers (Forward: 5'-TATATGAATTCATGGTTGATGACGATGGCAGCAT-3', Reverse: 5'-TATCTACTCGAGTTAATTACCATTTTCAGCTGTAACAGC-3'). Restriction mapping was conducted on PCR product with *Pst*I restriction enzyme. The PCR product was digested with *Eco*RI and *Hind*III and inserted into the pET32a (+) vector at the corresponding sites. The new construct was named pET32a (+)-AbBap. The transformants were grown in LB medium containing 80 µg ampicillin ml⁻¹.

2.3. Expression and purification of recombinant protein

E. coli BL21 (DE3) cells transformed with pET32a (+)-AbBap were grown in LB medium supplemented with 80 µg/ml ampicillin at 37 °C with shaking (220 rpm) to reach an OD₆₀₀ of 0.6, and then induced with 1 mM IPTG. The cells were collected 5 h after induction by centrifugation at 5000 g for 10 min. The cells were resuspended in lysis buffer containing 8 M Urea and subjected to sonication (4 times, 20 s at 200 w with a 10 min cooling period between each burst). The lysate was incubated for 2 h at 37 °C and then centrifuged at 14,000 g for 20 min at 4 °C to pellet the cellular debris. The supernatant was loaded on Ni-NTA agarose affinity column. The column was washed with Qiagen buffers C, D and E, (based on pH gradient of solutions). E buffer was used to elute the recombinant Bap subunit protein. All the buffer solutions contained 8 M urea. The protein was analyzed by 12% SDS-PAGE. The protein was refolded by sequential dialysis carried out against PBS (pH 7.4) containing 6, 4, 2 and 0 M urea respectively. Concentration of the purified protein was determined by Bradford method.

2.4. Western blotting

In order to confirm expression of the recombinant Bap subunit, western blotting was performed. 0.5 µg from each of the recombinant Bap subunit and uninduced transformed cell lysates (control) were separated by electrophoresis using 12% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane (Pore Size 0.45 µm – Invitrogen (using Bio-Rad mini-protein tetra system). The membrane was incubated in 5% skimmed milk in PBS–0.05% Tween-20 (PBS-T), for 12 h at room temperature. The membrane was washed 3 times with PBS-T and incubated in PBS buffer containing anti-His antibody conjugated with horseradish peroxidase

(Qiagen) at 1:8000 dilution for 1 h. The nitrocellulose paper was washed 3 times for 10 min each with PBS-T. The bands were visualized with diaminobenzidine (DAB) substrate and the reaction was stopped with addition of PBS.

2.5. Animals husbandry

Male BALB/c mice, 4–6 weeks old (16–22 g), were procured from the Razi Institute, Tehran, Iran. 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 [11]. The animal care protocol was approved by ethical committee, Shahed University.

2.6. Immunization of mice

Fifteen male mice in group one received two subcutaneous doses of 20 µg recombinant Bap subunit each at 2 week intervals. The initial vaccinations were emulsified with complete Freund's adjuvant (Razi institute, Tehran), and the next with incomplete Freund's adjuvant (Razi institute, Tehran). Blood samples were collected 7 days post-injections through infra-orbital plexus. An additional 5 BALB/c mice included in the control group received PBS and Freund's adjuvant.

2.7. ELISA with recombinant Bap subunit

Four micrograms of the recombinant protein in 100 µl coating buffer was used to coat each well in 96-well microtiter plates. Coating was achieved by incubation for 12–18 h at 4 °C. Wells were washed three times with (PBS-T), and were then blocked with 100 µl of PBS-T plus 5% skimmed milk for 1 h at 37 °C. The wells were washed 3 times with PBS-T and then serial dilutions of each serum ranging from 1:100 to 1:6400 were added to the wells in triplicate and incubated at 37 °C for 1 h. The plate was washed 3 times. 100 µl per well of Goat anti-mice IgG conjugated to Horseradish peroxidase (IgG1-Qiagen) diluted to 1:10,000 in PBS-T was added and the plate was incubated for 1 h at 37 °C. The wells were then washed 3 times with PBS-T and then incubated with 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate until a desired absorbance was reached. The reactions were stopped by addition of 100 µl 3 N H₂SO₄. Optical densities of the samples were measured at 450 nm using an ELISA plate reader.

2.8. Infection of mice with *A. baumannii*

LD₅₀ was determined for *A. baumannii* in BALB/c mice and all injections were at one log under LD₅₀. Mice were divided into three groups. Group one was immunized with recombinant Bap subunit. Fifteen mice in group two received three subcutaneous doses of 100 µl *A. baumannii* (10⁷ CFU) with PBS each at 2 week intervals. An additional 5 BALB/c mice included in group three serving as control group received PBS only. Blood samples were collected 7 days post-injections through infra-orbital plexus.

2.9. Whole cell ELISA

E. coli BL21 (DE3), *A. baumannii*, *S. enteritidis* and *S. aureus* were grown in LB medium until an optical density of OD₆₀₀ reached 0.4 corresponding to 10⁷ CFU per ml. The bacteria were sedimented by centrifugation and resuspended in carbonate coating buffer (0.05 M, pH 9.6). Each well received 100 µl of the appropriate bacterial suspension. Serial dilutions ranging from 1:50 to 1:6400

of sera from mice infected with *A. baumannii* and from mice immunized with recombinant Bap subunit were analyzed by the whole cells coated in ELISA plate wells. *E. coli* BL21 (DE3) and *S. enteritidis* coated wells served as a non biofilm producing negative control bacteria. Biofilm producing strain of *S. aureus* i.e. ATCC 6538 was used as non *Acinetobacter* bacterial control. *A. baumannii* ATCC 19606 was used as a standard strain in addition to *A. baumannii* Kh0060.

2.10. Human patient and serum samples

Informed consent was obtained prior to blood collection from the patients in the ICU of Imam Khomeini with a nosocomial pneumonia and/or bloodstream infection due to resistant *A. baumannii*. Sera samples were stored at -70°C .

2.11. ELISA test against sera of infected patients

Four micrograms per well of the recombinant Bap was used to coat the surface of 96-well microtiter plates as described earlier. The wells were washed with PBS-T and then the unoccupied sites were blocked with 5% skimmed milk in PBS. After washing the plates 3 times with PBS-T (200 μl per well), serial dilutions of each serum ranging from 1 to 1:128 were added to the wells in triplicate and incubated at 37°C for 1 h. Plate was washed 3 times and 100 μl of human secondary IgG antibody diluted 1:10,000 in PBS-T was added to each well and the plate was incubated for 1 h at 37°C . Plate was then washed 3 times with PBS-T and incubated with 100 μl per well of TMB substrate until a desired absorbance was reached. The reactions were stopped by the addition of 100 μl per well of 3 N H_2SO_4 . The optical density of the samples was measured at 450 nm using an ELISA plate reader.

2.12. Western immunoblotting

In order to confirm specificity of recombinant Bap subunit, western immunoblotting was performed. 0.5 μg the purified recombinant Bap subunit was loaded in two separate gels and separated by electrophoresis using 12% SDS-PAGE. The proteins were transferred onto a nitrocellulose membranes (Pore Size 0.45 μm – Invitrogen (using Bio-Rad mini-protein tetra system. The membranes were incubated in 5% skimmed milk in PBS–0.05% Tween-20 (PBS-T), for 1 h at room temperature. The membranes were then washed 3 times with PBS-T and incubated in PBS buffer containing sera of human patients infected with *A. baumannii* (at 1:5 dilutions) and control individual for 1 h separately. The human secondary antibody diluted to 1:10,000 with PBS-T was added to nitrocellulose papers and incubated for 1 h at 37°C . The nitrocellulose papers were washed 3 times for 10 min each in PBS-T. The bands were visualized with diaminobenzidine (DAB) substrate and the reaction was stopped with addition of PBS.

2.13. Statistical analysis

The data are expressed as mean \pm standard deviation (SD). Oneway ANOVA with post hoc Scheffé tests determined statistical significance of ELISA and whole cell ELISA results. *P*-values were calculated by Student's *t* test to determine the significance of differences among the experimental groups. Graphpad software was used for data analysis. *P*-values of <0.05 were considered as significant.

3. Results

3.1. PCR amplification

The size of PCR amplicon of the fragment encoding desired Bap epitope was 1620 bp. To analyze the amplification of the correct sequence, restriction mapping was performed using *pstI* enzyme. On 1% agarose gel, the fragment had appropriate size and digestion pattern.

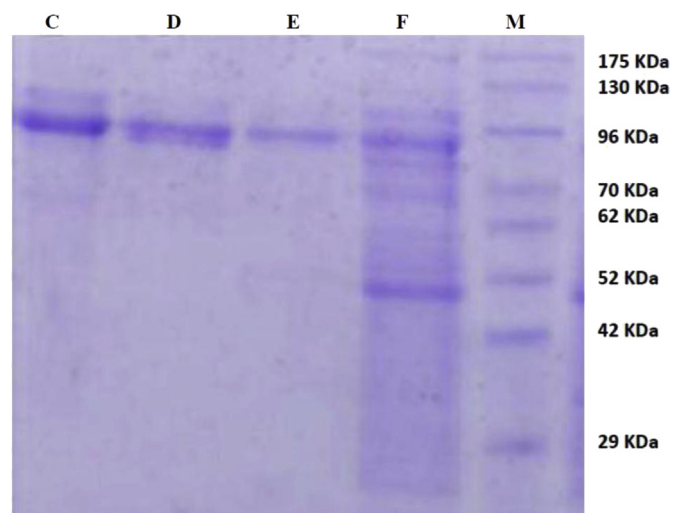
3.2. Expression and purification and western blot analysis of Bap subunit

The induction with 1 mM IPTG resulted in high expression of the recombinant protein. The recombinant protein was purified by Ni-NTA affinity chromatography. SDS-PAGE with Coomassie blue staining confirmed protein expression and purification. A single band of 78.4 kDa was obtained after purification (Fig. 1). Correct refolding of the recombinant Bap subunit was confirmed by indirect ELISA using sera of mice infected with the *A. baumannii* bacterium.

The recombinant Bap subunit was also confirmed by western blotting test. Transfer of samples from gels to nitrocellulose membranes was successfully observed by Ponceau S staining of nitrocellulose membrane. The HRP-coupled anti-polyhistidine antibodies were successfully fused to His-tag of Bap subunit and reacted with DAB. A 78.4 kDa band was observed in western blotting (Fig. 2).

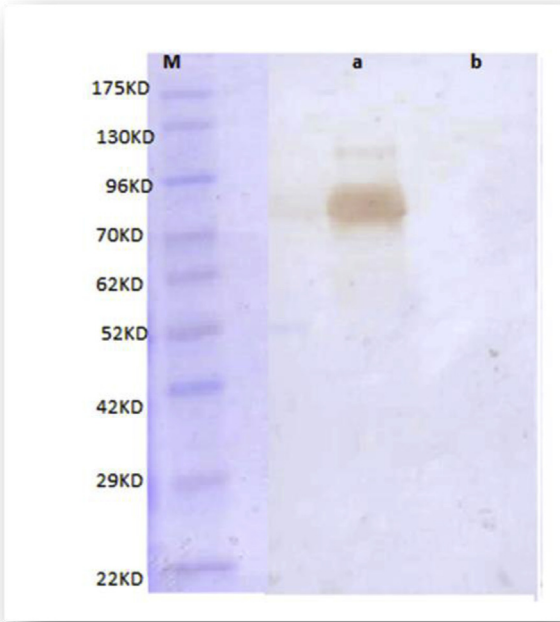
3.3. Antibody response against Bap subunit

The sera collected after first and second boosters were assessed for specific IgG production against Bap protein. Mice immunized with purified Bap subunit showed significant rise of IgG. Compared to the first dose, the antibody titer increased significantly ($P < 0.0001$) after the second booster, whereas the control group that received adjuvant and PBS showed no rise of antibodies in their sera (Fig. 3).



M: Standard protein marker; F: Cell lysate; C: Column washed with washing buffer; D & E: Columns washed with elution buffer. A single band of recombinant Bap subunit at approximately 78.4 kDa is seen in column E.

Fig. 1. SDS-PAGE image of a single band of 78.4 kDa purified recombinant Bap subunit produced in *E. coli* BL21DE3.



M: Standard protein marker; a: Purified recombinant Bap subunit; b: Uninduced transformed cells lysate.

Fig. 2. A 78.4 kDa band observed in western blotting as a result of successful fusion of HRP-coupled anti-polyhistidine antibodies to His-tag of Bap subunit.

3.4. Whole cell ELISA

Significant OD values were detected by ELISA using sera of the mice immunized with Bap in *A. baumannii* Kh0060 and *A. baumannii* ATCC 19606 coated wells. *E. coli* BL21 (DE3), *S. enteritidis* and *S. aureus* coated wells serving as the negative control showed no immunoreactivity with same immunized sera. *A. baumannii* Kh0060 coated wells were also tested with sera obtained from control mice and showed no rise in the OD (Fig. 4).

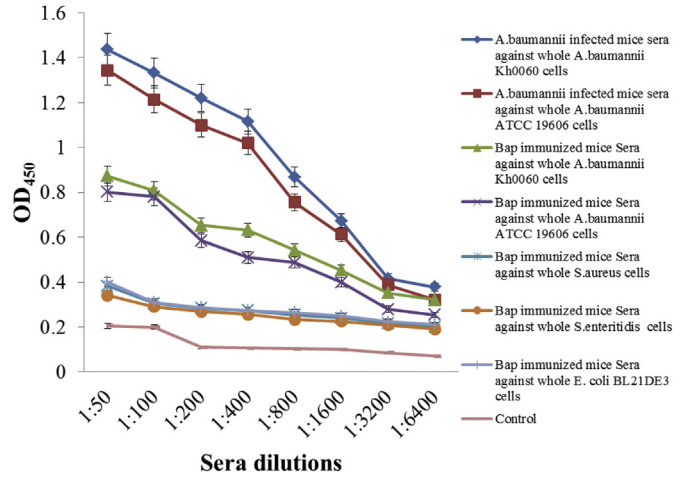


Fig. 4. Whole cell ELISA results, cross reaction of sera from Bap subunit and *A. baumannii* immunized mice against *A. baumannii* (Kh0060).

3.5. ELISA test against sera of patients infected with *A. baumannii*

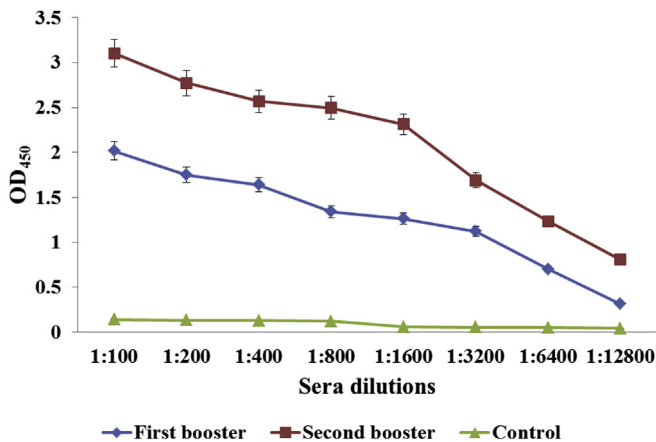
In comparison with control serum significant OD values were detected when recombinant Bap subunit was tested with sera from human patients infected with *A. baumannii*. The sera obtained from control healthy human showed no rise in the OD (Fig. 5).

3.6. Western immunoblot analysis

Specificity of the recombinant Bap subunit was confirmed by western immunoblotting test. Transfer of samples from gels to nitrocellulose membranes were successfully observed by Ponceau S staining of nitrocellulose membrane. Sera obtained from infected patients successfully attached to recombinant Bap protein and resulted in a 78.4 kDa band on a nitrocellulose paper whereas control sera did not result in any reaction (Fig. 6).

4. Discussion

Nosocomial infections caused by *Acinetobacter* spp. have increased in recent years [12]. Pandrug-resistant (PDR) and extremely drug-resistant strains of *A. baumannii* have recently been reported [13]. The limitations in treatment of *Acinetobacter*



The sera were collected after first and second booster injections and assessed for specific anti-Bap IgG. Antibody titers increased significantly (P value <0.0001) in the second booster. Unimmunized mice sera were used as control.

Fig. 3. Indirect ELISA showing significant rise of IgG in sera of mice immunized mice with Bap subunit.

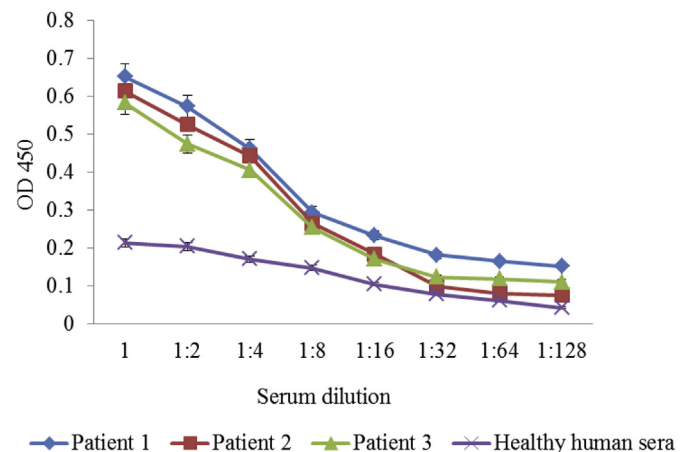
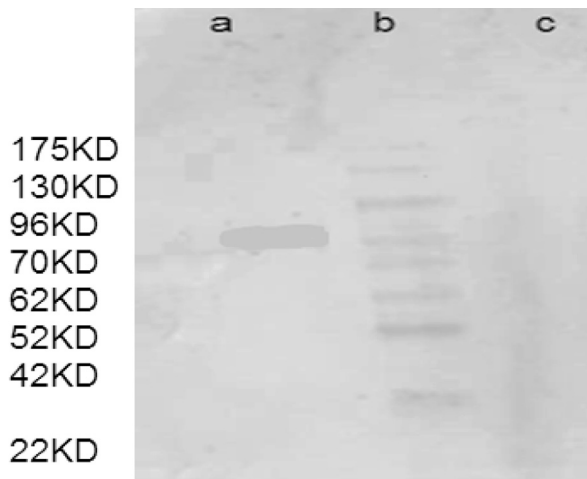


Fig. 5. Indirect ELISA of recombinant Bap subunit protein against *A. baumannii* infected human serum. Sera from healthy individuals were used as control.



a: Infected human serum; b: Standard protein marker; c: Healthy human serum (Negative control).

Fig. 6. Specificity of the recombinant Bap subunit confirmed by western immunoblotting.

infections or lack of therapeutic options require use of new treatment routes other than the classical antibiotic treatment [14]. Bap-related proteins are outer membrane proteins, but it has also been suggested that Bap could be an extracellular protein [15] involved in intercellular adhesion within the mature biofilm and plays a significant role in bacterial infections processes. Bioinformatics studies suggested 15 potential linear B-cell epitopes within the primary sequence of the Bap subunit and homology search of these sequences returned mostly to *A. baumannii* [5]. Higher scores and the lowest BLAST hits for this subunit both belong to *A. baumannii* and shows the specificity of this region to the bacterium [9]. Number and arrangement of repeat units are the main differences between Bap homologs [5]. Since this subunit is conserved and specific within *A. baumannii* strains, it could be a target for development of a diagnostic tool to detect bacterial infections. BLAST data show a few homologies of this subunit to the proteins of *S. enteritidis*. The Eliza data confirmed these bioinformatic findings. Available commercial systems are not satisfactory in diagnosis of *Acinetobacter* species. In order to accurately identify, genotypic identification methods have been used including DNA polymorphism analysis based on selective amplification of restriction fragments (AFLP analysis) and also detection based on 16 S rDNA restriction analysis (ARDRA). These methods are limited in few reference laboratories and are not used in routine clinical laboratories [3]. Detection of this organism is still carried out by conventional microbiology and biochemical tests which are time consuming and costly. Rapid emergence, prevalence and rise of pan-resistant antibiotic strains is an indication of a need for a fundamental diagnostic test that is rapid, sensitive and specific as compared to the conventional detection methods. Development of such test would require finding specific antigenic proteins of *A. baumannii* [5]. Failure of routine clinical identification methods of *A. baumannii* isolates leads to use of more expensive tests and delay in diagnosis time that could have significant impact on treatment [3]. The interaction between serum infected mice with *A. baumannii* with Bap and vice versa can be demonstrated natural refolding Bap. Gundi et al. [3], identified a 350 bp highly variable zone on the rpoB gene which appeared to be a promising target for identification of *Acinetobacter* species. The disadvantage of their method is constant pipetting and a need of transportation and

storage in cold chain. Use of immune based assays is becoming more routine and reliable in identification of microbial agents, Bettina Wilske and co-workers [15] reported that pC is expressed on the outer surface of *Borrelia burgdorferi*. They used this protein to detect *B. burgdorferi* infection. Use of immunoserologic tests for diagnostics of borreliosis has been reported [16]. In this study sera from patients infected with *A. baumannii* were successfully tested against recombinant Bap epitope. Sera from healthy individuals did not show a reaction in ELISA or Western immunoblotting. These results support the earlier bioinformatics studies and helps diagnosis of *A. baumannii* infection based on immunoserologic tests. In conclusion since Bap is specific and conserved within *A. baumannii* strains, outer membrane proteins (OMPs), hence the conserved regions from Bap can be used as a biomarker in the development of diagnostic tests for *A. baumannii* detection.

Acknowledgment

The authors wish to thank Shahed University, Iranian Ministry of health and Biotechnology Development Council of I. R. Iran for supporting the conduct of this research. The Authors declare that there is no conflict of interest.

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