Antibiotic Characterization of *Acinetobacter baumannii* Isolated from Clinical Samples and Production of Recombinant OmpA from Resistant Strains

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Abstract

Background: *Acinetobacter baumannii* is one the most dangerous microbes that is resistant to a wide range of prescribed antibiotics, thus development of more effective approaches is urgent. Recombinant protein vaccines have been recently proposed as a safe method to cope with infectious agents. In this approach, antigenic gene of a bacterium can be cloned in an expression vector to produce recombinant protein.

Objectives: In this study, the prevalence of *A. baumannii* was determined in hospitalized samples and the antimicrobial susceptibility patterns of isolates were characterized for a variety of antibiotics. The *ompA* gene from resistant isolates was then amplified and cloned in an expression vector to produce the recombinant OmpA protein in *Escherichia coli*.

Methods: The clinical samples were collected from sputum, wounds, septicemia, and urinary tract infections at the intensive care unit (ICU) and different wards of hospitals and *A. baumannii* were identified, according to standard diagnostic tests. The antibiotic susceptibility patterns of the isolates were determined for 17 antibiotics. The *ompA* gene of *A. baumannii* was amplified using the polymerase chain reaction (PCR). The *ompA*-amplicon was cloned and sub-cloned in pTZ57RT and pET32a (+) vectors, respectively. Double digestion, DNA sequencing, and PCR were performed to confirm that *ompA* has been truly cloned. Using RT-PCR and SDS-PAGE, the expression of recombinant OmpA was assessed in IPTG-induced E. coli.

Results: Most of the *A. baumannii* were resistant to antibiotics cefepime, ceftazidime, ceftriaxone, aztreonam, amikacin, and gentamycin, and the least resistance was found towards colistin, ampicillin-sulbactam, and trimethoprim antibiotics. The *ompA* gene was amplified as 1112 bp amplicon, which was successfully cloned and sub-cloned in the pTZ57RT-T/A and pET32a (+) vectors. The presence of ~38 kDa band in SDS-PAGE showed that the recombinant pET32a-*ompA* was highly expressed in the host cells.

Conclusions: The obtained data showed the high resistance of *A. baumannii*-infected isolates to the antibiotics. Besides, successful cloning and expression of rOmpA in the cells suggests that the antigenic properties of *ompA* gene may be considered in future researches.

Keywords: *Acinetobacter baumannii*, Antibacterial Agent, Multidrug Resistant, OMPA Outer Membrane Protein

1. Background

Acinetobacter baumannii are non-motile Gram-negative bacteria, which can be found widespread in soil, water, and various samples of humans or animals (1, 2). These bacteria are a leading cause of endocarditis, skin and soft tissue infections, meningitis, urinary tract infections, and pneumonia (3, 4), partly due to resistant features of this opportunistic microbe to a variety of antibiotics, resulting in rapid emergence of multidrug and extremely drug resistant strains (5). This is the reason these bacteria have become one the most successful pathogens in the modern healthcare system and endemic in hospitals, especially in patients at the intensive care unit (ICU) (6). Additionally, *A. baumannii* can survive in both dry and damp hospital environmental conditions, even for lengthened periods of time (1, 7). Therefore, development of non-antibiotic-based intervention strategies seems urgent (8). A variety of risk factors have been proposed in acquiring *A. baumannii* infection. These include immunosuppression, unscheduled hospital admission, respiratory failure at ICU admission, previous antimicrobial therapy, previous sepsis in the ICU, and the invasive procedures index (9).

Increase of the number of patients with acquired nosocomial *Acinetobacter* infectious outside the ICU has been recently reported, which is alarming and shows the ability of this pathogen to become a major healthcare barrier (10). These findings suggest urgent need for the development of novel strategies to fight this multidrug-resistant pathogen (11). Vaccines are one of the most effective medical plans for the control of infections worldwide and are usually designed based on weekend bacteria or their purified immunogenic parts, such as proteins or polysaccharide wraps. However, such vaccines are not very effective for inducing cell-mediated immunity against intracellular pathogens (12, 13). Recently, a new strategy has been proposed, which is based on the production of recombinant protein from infectious agents with acceptable antigenic potential. In this approach, which is also known as second generation of vaccines, a gene encoding antigenic protein can be cloned in an expression plasmid, expressed in a host cell, including bacteria or yeast.

The recombinant protein was then purified to be tested as a vaccine (13). Such strategy has been recently proposed and applied to develop hepatitis B virus (HBV), human papillomavirus (HPV), human immunodeficiency virus (HIV), tuberculosis (TB), and malaria vaccine (14). Besides, clinical trials have shown that these vaccines are safe and well-tolerated (15). In Gram-negative bacteria, the outer membrane is a safeguard layer against external perilous agents, such as foreign enzymes and detergents (16). It has been documented that outer membrane proteins (OMPs), such as OmpA, promote the virulence features of *A. baumannii* through their critical role in biofilm formation or bacterial adhesion on epithelial cells (2, 17). These proteins can also cause bacteria to withstand against environmental stress (18). In a reverse vaccinology study by Moriel, the *ompA* gene was introduced as one the potent genes to develop DNA vaccine against *A. baumannii* (19).

2. Objectives

It has been indicated that OmpA are a potent inducer of native and adaptive immune responses *in vivo* (20). Notably, 2% to 3% of the Gram negative bacteria genome consists of genes encoding the OMPs. Therefore, these proteins are recently proposed as proper candidates for recombinant vaccine development (21). Here, the *ompA* gene of *A. baumannii* was extracted from antibiotic resistant *A. baumannii*-infected clinical samples and efficiently cloned and expressed in pET32a (+) expression system, one of the most effective systems for gene expression and production of recombinant proteins (22).

3. Methods

3.1. Ethics Statement

This study (experimental and cross-sectional) was part of a Ph.D. thesis, which was ethically approved by the Islamic Azad University of Marvdasht, Iran (IR.MIAU.REC.1397.802), and experimentally performed at Islamic Azad University of Shahrekord, Iran.

3.2. Bacterial Isolates

Sixty-seven samples were collected from different clinical cases of pneumonia (sputum, n = 25 isolates), wound pus (n = 18 isolates), septicemia (blood, n = 14 isolates), and urinary tract infections (n = 10 isolates) at the ICU and different wards of Imam Ali and Kashani hospitals of Shahrekord, southwest Iran, during September 2015 to April 2016. To avoid multiple entries from a single patient, only the first sample for a given patient was included. All samples were cultured on solid media (MacConkey; Merck, Germany and blood agars). The *A. baumannii* was diagnosed at a microscopic scale as well as biochemical and bacteriological standard tests. The two later assays included growth at 44°C, Gram staining, MacConkey agar, Sulfur Indole Motility media (SIM) assay, oxidase-catalase assay, hemolysis test, triple sugar iron (TSI) test, arginine hydrolysis assay, ornithine decarboxylase test, citrate assay, and gelatin hydrolysis. The isolates were stored at -70°C in trypticase soy broth (Merck, Germany), supplemented with 15% glycerol, before use.

3.3. Antimicrobial Susceptibility Tests

The disk diffusion method was used to examine the susceptibility of all isolates to 17 antimicrobial agents, as recommended and outlined by the National Committee for Clinical Laboratory Standards (CLSI, 26th edition 2016, USA) (23). Antimicrobial agents, including tobramycin (10 μg), tetracycline (30 μg), oxacillin (1 μg), trimethoprim (5 μg), gentamicin (10 μg), meropenem (10 μg), imipenem (10 μg), colistin (10 μg), ceftriaxone (30 μg), ampicillin-sulbactam (10/10 μg), co-trimoxazole (30 μg), ciprofloxacin (5 μg), cefepime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), amikacin (30 μg), and piperacillin-tazobactam (100/10 μg) were used in this study. All of the antibiotics were purchased from MAST, UK or Liofilichem, Italy. The isolates were grown on trypticase soy agar plates, supplemented with 5% sheep blood at 37°C overnight. The *A. baumannii* ATCC 19606 and *Escherichia coli* ATTC 25922 were purchased from the bacterial repository of Iranian Biological Resource Center (IBRC, Karaj, Iran) and considered for quality control purposes (positive and negative control) in the antibacterial susceptibility assay.

3.4. Detection of MDR, PDR and XDR Isolates

Defining of multidrug resistance (MDR), pan drug resistant (PDR), and extreme drug resistance (XDR) in *A. baumannii* isolates was done according to new standardized international document (24), with the results of antimicrobial susceptibility of *A. baumannii* to all antimicrobial agents listed in Table 1. Briefly, MDR is defined as non-susceptibility to ≥ 1 agent in ≥ 3 antimicrobial categories, XDR was described as non-susceptibility to ≥ 1 agent in ≤ 2 antimicrobial categories, and PDR was defined as non-susceptibility to all antimicrobial agents listed.

3.5. Extraction of Genomic DNA from Acinetobacter baumannii

The first step to development of the *ompA*-containing construct was the extraction of genomic DNA from resistant isolates, using the DNA extraction kit (Qiagen DNA Mini Kit, Iran), according to manufacturer's instructions. The integrity of extracted DNA was evaluated through 1% agarose gel electrophoresis. The purity and concentration of DNA samples was calculated by Nano-Drop ND-1000 (PeqLab Ltd., UK) at wavelengths of 230, 260, and 280 nm.

3.6. Polymerase Chain Reaction (PCR)

The *ompA* gene (accession number: AY485227) coding sequence was amplified using specific primers, as the 1112 bp product. The primer sequences were designed using Gene Runner software version 5. The sequences of primers was as follow: Forward: 5'-AGGAAGCTTATGAAATTGAGTCGTATTGC-3' and reverse: 5'-CGTCTCGAGTTTTTACTGTTCAAGAACTC-3'. A restriction site for *HindIII* and *XhoI* were included in forward and reverse primers, respectively. The PCR reaction was optimized in a total volume of 25 μL, containing 2.5 μL 10X PCR buffer, 2 mM MgCl$_2$, 100 mM dNTPs, 1 mM of each primer, 1 μg DNA sample, and 1 unit of *Taq* DNA polymerase (Fermentas, Germany). The PCR was programed as 35 cycles of initial denaturation at 95°C for five minutes; denaturation at 94°C for one minute, annealing at 61°C for one minute, extension 72°C for one minute, and final extension at 72°C for five minutes. The PCR products were checked on 2% agarose gel electrophoresis and bands were visualized under UV trans-illuminator (Uvtec, UK).

3.7. Cloning ompA Gene

The *ompA* amplicon were purified from the gel, using the gel extraction kit (Bioneer, Korea), according to the manufacturer's instructions and used for cloning in T/A cloning vector pTZ57RT (Thermo Scientific, Lithuania). The recombinant construct was then subjected to transform *E. coli NovaBlue* competent cells, using 42°C heat shock and calcium chloride method. Transformed competent cells were then cultured in LB agar media (Merck Co., Germany), containing 20 mg/mL X-gal, 0.1 M IPTG, and 100 μg/mL ampicillin at 37°C overnight. The white colonies were re-cultured in LB broth media, supplemented with 100 μg/mL ampicillin at 37°C overnight. The recombinant vector was extracted from bacterial cells using Plasmid Mini Extraction Kit (Bioneer, Korea), according to the user manual. The *HindIII/XhoI* double digestion was performed to confirm whether *ompA* has been correctly inserted in the vector. Polymerase chain reaction-sequencing was also applied to validate the accurate cloning.

3.8. Sub-Cloning of ompA Gene in the Expression Vector pET-32a (+)

Using *HindIII/XhoI* double digestion, the 1112 bp corresponding to *ompA* amplicon was removed from the T/A recombinant pTZ57RT vector and considered to ligate with *HindIII/XhoI* linearized pET-32a. The T4 ligase enzyme was used to generate the pET-32a-*ompA* recombinant expression vector. The *E. coli NovaBlue* competent cells were used as a transformation host, which were cultured in LB agar media, containing 100 μg/mL of ampicillin. The pET-32a-*ompA* recombinant construct was validated by PCR-sequencing, using *ompA*-F and *ompA*-R primers, as well as *HindIII/XhoI* double digestion.

3.9. SDS-PAGE Analysis

The pET32a-*ompA* was introduced in the *E. coli Rosetta-gami™ 2 (DE3)*, using the CaCl$_2$ method. Transformed cells were then cultured on the LB agar, containing 100 μg/mL ampicillin and 35 μg/mL chloramphenicol. The recombinant *E. coli DE3* was grown in 5 mL LB broth, containing ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL) on a shaking incubator overnight at 37°C. Then, 500 mL of culture was inoculated in 50 mL of LB broth medium at 37°C with agitation at 200 rpm. The cells were grown until the optical density reached 0.6 to 0.8 at 600 nm. Expression of the OmpA protein was induced by the 0.9 mM IPTG and incubated for five hours at 250 rpm/min, at 37°C. The cells were harvested by centrifugation at 9000 rpm. The harvested cells then subjected for sonication; Ten short bursts of 10 seconds followed by intervals of 30 seconds on ice to obtain the viscous cell lysate. Whole cell lysate was then analyzed on 12% SDS-PAGE. The IPTG-induced *E. coli* with empty pET32a was also considered as the negative control. The mentioned control confirms that the empty vector does not express any proteins with similar molecular weight to the target protein (OmpA).

Table 1. Disk Diffusion Susceptibility Assay for 30 *Acinetobacter baumannii* Positive Isolates Collected from 67 Patients[a]

Antibiotic	S	I	R
Penicillins			
Ampicillin-Sulbactam (SAM)	16 (53.34)	4 (13.33)	10 (33.33)
Piperacillin plus Tazobactam (TZP)	7 (23.33)	4 (13.33)	19 (63.34)
Oxacillin (OXA)	4 (13.33)	9 (30)	17 (56.67)
Cephalosporins			
Ceftazidime (CAZ)	1 (3.33)	2 (6.67)	27 (90)
Ceftriaxone (CRO)	1 (3.33)	4 (13.33)	25 (83.34)
Cefepime (CPM)	0 (0)	2 (6.67)	28 (93.33)
Carbapenems and β-lactams			
Imipenem (IPM)	10 (33.33)	3 (10)	17 (56.67)
Meropenem (MEM)	5 (16.67)	9 (30)	16 (53.33)
Aztreonam (ATM)	4 (13.33)	3 (10)	23 (76.67)
Tetracyclines			
Tetracycline (TET)	9 (30)	6 (20)	15 (50)
Aminoglycosides			
Amikacin (AMK)	5 (16.67)	4 (13.33)	21 (70)
Tobramycin (TOB)	4 (13.33)	8 (26.67)	18 (60)
Gentamicin (GEN)	7 (23.23)	3 (10)	20 (66.67)
Sulphonamides			
Co-trimoxazole (TS)	4 (13.33)	9 (30)	17 (56.67)
Folate pathway inhibitor			
Trimethoprim (TP)	5 (16.67)	12 (40)	13 (43.33)
Quinolones			
Ciprofloxacin (CIP)	3 (10)	7 (23.33)	20 (66.67)
Lipopeptides			
Colistin (CO)	23 (76.67)	4 (13.33)	5 (10)

[a]Values are expressed as number (percentage).

3.10. RNA Extraction

Total RNA was extracted from 10^7 recombinant *E. coli DE3* containing pET32a-*ompA*, induced by IPTG, using RNX-Plus reagent (SinaClon, Iran), according to the user manual. The concentration and purity of the genomic DNA were evaluated by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), reading at wave length 230 nm, 260 nm, and 280 nm. The RNA was considered pure if the absorbance ratio of 260 nm/280 nm and 260 nm/230 nm was around two. The integrity of extracted RNAs was also evaluated on 1% agarose gel electrophoresis.

3.11. Complementary DNA (cDNA) Synthesis and RT-PCR

A reaction containing 500 ng/μL RNA, 100 mM dNTPs, 10 μL 5x RT-PCR buffer, 200 mM oligdT primer, and 1 U/μL M-MuLV reverse transcriptase, (Invitrogen, USA) in a total volume of 20 μL was prepared for cDNA synthesis. The mixture was incubated at 42°C for one hour, followed by 15

minutes at 70°C for enzyme inactivation. The *ompA* transcript was then amplified using the specific primers, as mentioned above. The reaction was performed in total volume of 20 μL, containing 50 mM MgCl$_2$, 0.25 mM of each dNTP, 2 pmol/L of each primer, 400 ng/μL cDNA templates, and 1 U of Taq DNA polymerase (Fermentas, Germany). The final volume was adjusted with nuclease-free water. The PCR was programmed in a thermocycler as five minutes of denaturation at 95°C for initial denaturation, followed by 25 cycles of denaturation; 94°C for one minute, annealing; 61°C for one minute, extension; 72°C for 45 seconds, final extension; 72°C for two minutes. A sample without DNA template (NTC) was included in all PCR reactions as the negative control. The PCR products were then analyzed on 1% agarose gel electrophoresis, dyed with 2% ethidium bromide, and product bands were visualized under ultraviolet light.

3.12. Statistical Analysis

All the data was entered in the SPSS version 22 (SPSS Inc., Chicago, IL) software for descriptive analysis. A two-tailed P < 0.05 was considered statistically significant. In susceptibility assay, data were classified as susceptible and non-susceptible (including sensitive; S, intermediate; I and resistant groups; R).

4. Results

4.1. Antibiotic Resistance Pattern of Acinetobacter baumannii

Out of 67 collected samples, 30 samples (44.48%) were diagnosed as A. baumannii positive. These bacteria were recovered from sputum (n = 15 isolates), wound pus (n = 7 isolates), blood (n = 6 isolates), and urine (n = 2 isolates). These 30 isolates were subjected to antibiotic susceptibility assay, the data of which has been summarized in Table 1. This study found that these 30 bacterial isolates were highly resistant to all antibiotics, except colistin and ampicillin-sulbactam. In this way, 66.7% to 93.3% of the strains were resistant to the antibiotics cefepime, ceftazidime, ceftriaxone, aztreonam, amikacin, gentamicin, and ciprofloxacin. The distribution of resistant isolates in different samples is summarized in Table 2. As illustrated in Table 3, 29 isolates were MDR and one isolate was resistant to all categories of tested antibiotics, which places it in the PDR group.

4.2. The Acinetobacter baumannii-Associated ompA Gene Was Successfully Cloned and Sub-Cloned inpTZ57RT and pET32a (+) Vectors

The ompA gene was amplified using PCR, as 1112 bp product (Figure 1). The amplicon was then subjected to cloned in the pTZ57RT vector. The PCR-DNA sequencing confirmed that the inserted ompA was completely aligned with the 98 recorded sequence of ompA for A. baumannii in gene bank. The degree of identity varied from 98% to 100%. The list of isolates, which had maximum sequence identity with the ompA sequence of the present study has been presented in Supplementary File. The obtained data from HindIII and XhoI double digestion also confirmed that ompA was correctly incorporated in the pTZ57RT vector. The presence of bands 1112 bp and 2886 bp during the electrophoresis was consistent with this fact. These bands belonged to the target gene and vector, respectively (Figure 2). Consequently, 1112-bp fragment of ompA was directly cloned at the HindIII and XhoI site of pET-32a, resulting in a recombinant plasmid, named pET-32a(+)-ompA. The recombinant plasmid (pET-32a(+)-ompA) was confirmed by PCR and HindIII/XhoI double digestion. The obtained data showed that the recombinant plasmid contained the ompA

target gene. The bands 1112 bp and 5900 bp were observed during the electrophoresis, as expected. These bands were related to the target gene and vector, respectively (Figures 3A and 3B).

4.3. The rOmpA Protein Was Expressed in the E. coli Through Recombinant pET-32a(+)-ompA Construct

The pET-32a(+)-ompA construct was transferred to E. coli Rosetta-gami and expression of recombinant protein was evaluated through IPTG induction. As illustrated in Figure 4, the presence of a ~38 kDa protein band (as expected for OmpA protein) on SDS-PAGE showed that the pET32a vector was capable of expressing the OmpA protein.
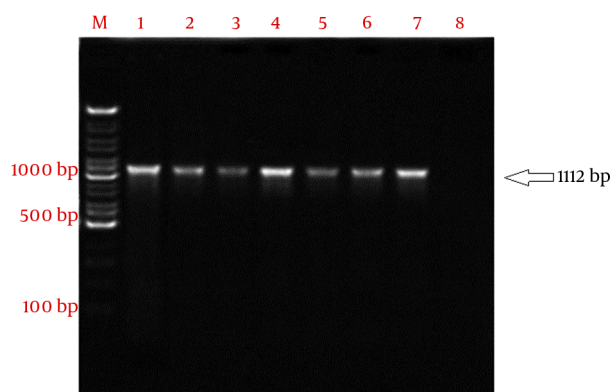
Figure 1. The PCR amplification of ompA gene from Acinetobacter baumannii isolates. Lane M, 100 bp DNA marker; lane 1 - 7, 1112 bp amplified ompA gene from A. baumannii strain; lane 8, negative control.
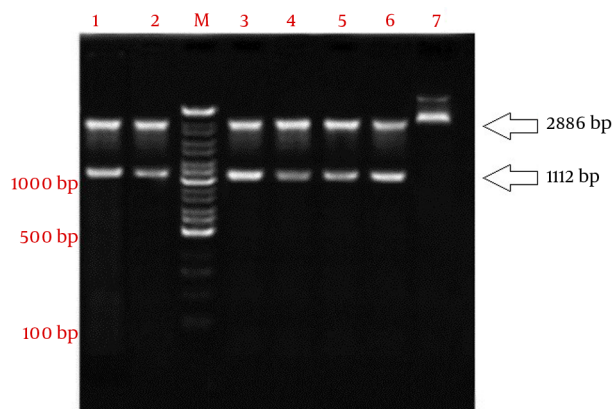
Figure 2. Analysis of recombinant plasmid (pTZ57RT-ompA) digested by two restrictive endonucleases HindIII and XhoI. Lane M, 100 bp DNA marker; lane 1 - 6, 2886 bp pTZ57RT fragment and 1112 bp ompA amplicon; lane 7, pTZ57RT-ompA undigested.

Table 2. Disk Diffusion Susceptibility Assay for 30 *Acinetobacter baumannii* Positive Isolates Collected from 67 Patients Based on the Sample Source[a]

Source	SAM	AMK	ATM	CAZ	CPM	CIP	TS	CRO	CO	TOB	IPM	MEM	GEN	TP	OXA	TET	TZP
Septum (n = 25)																	
S	3	1	0	1	0	1	2	1	18	1	4	1	3	2	1	2	3
I	1	0	0	1	0	2	3	0	2	2	1	4	0	5	4	1	1
R	0	2	2	2	6	1	1	10	1	4	1	2	7	5	2	0	13
Wound pus (n = 18)																	
S	3	1	1	0	0	1	1	0	3	1	3	2	2	1	1	3	1
I	0	1	1	0	0	1	2	1	1	4	1	5	1	3	2	3	1
R	1	2	4	8	5	3	3	2	2	6	6	3	5	6	3	0	4
Septicemia blood (n = 14)																	
S	5	2	0	0	0	0	1	0	1	1	1	2	2	1	1	3	1
I	1	0	1	0	1	3	2	2	0	1	1	0	1	1	1	0	1
R	4	4	8	8	6	10	10	6	2	0	6	3	5	5	2	7	2
Urinary tract infection (n = 10)																	
S	5	1	2	0	0	1	0	0	1	1	2	0	0	1	1	1	2
I	1	2	1	1	1	1	1	1	1	3	0	0	1	3	1	2	1
R	5	13	9	9	11	6	3	7	0	8	5	8	3	4	10	8	0

[a] Data has been presented as the number of isolates in each group.

4.4. The ompA-pET32a Was Successfully Expressed in Recombinant E. coli

The RNA was extracted from the recombinant *E. coli*, containing pET32a-*ompA* induced by IPTG. The cDNA was then synthesized from the transcripts to be amplified in RT-PCR, using specific primers of *ompA*. The presence of 1112 bp band confirmed that the pET32a vector can express the *ompA* coding sequence (Figure 5).

5. Discussion

Over the past two decades, *A. baumannii* was reported as one the major health problems due to the emergence of resistant strains, making it a dangerous opportunistic nosocomial pathogen (25). In a commentary published by the Center for Disease Control and Prevention during year 2004, *A. baumannii* was introduced as the leading cause of 80% of nosocomial infections and second pathogen isolated from hospitalized patients after *Pseudomonas* strains (26). Nosocomial septicity caused by the *A. baumannii* strains are progressively becoming resistant to a wide range of antibiotics (27). The current study revealed the resistant pattern of 30 strains of *A. baumannii* isolated from 67 clinical samples. All of the included patients were hospitalized at the ICU or different wards of Shahrekord hospitals. The samples were collected from sputum, urine, wound pus, and septicemia although most of them were obtained from sputum culture.

It was found that all of the 30 isolates of *A. baumannii* were resistant to examined antibiotics, except colistin and ampicillin-sulbactam. Most of the isolates were resistant to antibiotics cefepime, ceftazidime, ceftriaxone, aztreonam, amikacin, gentamicin, and ciprofloxacin, especially to the three antibiotics cefepime, ceftazidime, and ceftriaxone. This finding indicates that colistin and ampicillin-sulbactam may be the most effective antibacterial agents against *A. baumannii* infections and of course better choices for their treatment. Similarly, in a recently published study by Mohajeri et al. on 104 samples of attending patients of Kermanshah hospital, they found that all isolates had high-level of resistance to imipenem and meropenem, while no resistance to polymyxin B, colistin, tigecylcine, and minocycline was observed (28). In a study by Rahbar et al., the resistance pattern of 88 strains of *A. baumannii* was studied from clinical specimens, and high rate of resistance to ceftriaxone (90.9%), piperacillin (90.9%), ceftazidime (84.1%), amikacin (85.2%), and ciprofloxacin (90.9%) was indicated (29).

In 2011, Vahdani et al. examined the resistance pattern of 101 strains of *A. baumannii*, isolated from hospitalized patients, and found that isolates were resistant to ceftazidime (96%), ceftizoxime (95%), ceftriaxone (93%), amikacin (58%), gentamicin (68%), co-terimoxazole (85%), and ciprofloxacin (85%) (30). Previously published experiments showed that *A. baumannii* strains are highly resistant to third and fourth generation of cephalosporins, carbapenems, quinolones, and aminoglycosides, such as cefepime, ceftazidime, ceftriaxone, aztreonam, amikacin, gentamicin, and imipenem, although some differences exist (31, 32). Variable resistance patterns in different popula-

Table 3. Thirty Antibiotic Resistance Patterns of *A. baumannii* Found in 67 Clinical Samples Collected from Patients with Nosocomial Infections, in Different Wards of Hospitals

Antibiotics Resistant Patterns	Number of Antibiotics in the Resistant Pattern	Sample No.	Frequency in 30 Isolates	Type of Resistant Pattern
CPM, CAZ, CRO, ATM, AMK, CIP, TZP, TOB, TS, IPM, OXA, MEM, TET, SAM	14	1	1	MDR
CPM, CAZ, CRO, ATM, AMK, GEN, CIP, TZP, TOB, TS, IPM, OXA, MEM, TET, TP, SAM, CO	17	2	1	PDR
CPM, CAZ, CRO, ATM, AMK, GEN, CIP, TZP, TOB, TS, OXA, MEM, TET, TP	14	3	1	MDR
CPM, CAZ, CRO, ATM, AMK, CIP, TZP, OXA, TET, TP	10	4	1	MDR
CPM, CAZ, GEN, TZP, TOB, TS, IPM, TP	8	5	1	MDR
CPM, CAZ, CRO, AMK, GEN, TZP, TS, OXA, SAM	9	6	1	MDR
CPM, CRO, ATM, AMK, CIP, TZP, MEM	7	7	1	MDR
CPM, CAZ, CRO, ATM, GEN, TZP, TOB, IPM, OXA, TET, SAM	11	8	1	MDR
CPM, CAZ, CRO, ATM, TZP, TOB, TS, IPM, TET, TP	10	9	1	MDR
CPM, CAZ, ATM, AMK, CIP, TOB, IPM, SAM	8	10	1	MDR
CPM, CAZ, CRO, CIP, TS, IPM, OXA, TP, SAM	9	11	1	MDR
CPM, CAZ, CRO, ATM, AMK, GEN, TZP, IPM, MEM, CO	10	12	1	MDR
CPM, CRO, ATM, AMK, GEN, CIP, TOB, TS, OXA, TET	10	13	1	MDR
CPM, CAZ, CRO, CIP, TZP, TS, TP, SAM	8	14	1	MDR
CPM, CAZ, CRO, ATM, AMK, TZP, IPM, OXA, MEM, SAM	10	15	1	MDR
CPM, CAZ, CRO, AMK, GEN, CIP, TS, OXA	8	16	1	MDR
CPM, CAZ, ATM, AMK, GEN, TOB, TS, MEM, TET	9	17	1	MDR
CPM, CAZ, CRO, AMK, GEN, TZP, TOB, IPM, MEM, TP, SAM, CO	12	18	1	MDR
CAZ, CRO, ATM, GEN, CIP, TS, OXA, MEM	8	19	1	MDR
CPM, CAZ, AMK, GEN, CIP, TZP, TOB, IPM, MEM, TP	10	20	1	MDR
CPM, CAZ, ATM, AMK, GEN, CIP, TZP, TOB, TS, IPM, OXA, MEM	12	21	1	MDR
CPM, CRO, ATM, AMK, GEN, CIP, TOB, TS, OXA, MEM, TET	11	22	1	MDR
CPM, CAZ, CRO, ATM, GEN, CIP, TZP, IPM, MEM, SAM	10	23	1	MDR
CPM, CAZ, CRO, ATM, AMK, CIP, TS, IPM, MEM, TET, TP, SAM	12	24	1	MDR
CPM, CAZ, CRO, ATM, AMK, CIP, TZP, TOB, OXA, MEM, TP	11	25	1	MDR
CAZ, CRO, ATM, AMK, GEN, TS, OXA, TET, TP	9	26	1	MDR
CPM, CAZ, CRO, ATM, GEN, CIP, TOB, IPM, MEM, TET, TP	11	27	1	MDR
CPM, CAZ, CRO, ATM, AMK, GEN, TZP, TOB, IPM, TET	10	28	1	MDR
CPM, CAZ, CRO, ATM, AMK, GEN, CIP, TOB, TS, OXA, TET	11	29	1	MDR
CPM, CAZ, CRO, ATM, GEN, CIP, TZP, TOB, IPM, OXA, TET	11	30	1	MDR

tions might be partly due to hospital-associated healthcare settings available to patients, physician's experience, medical care, and supportive care (33, 34).

As an example, in some studies, high percentage of resistance was related to cefotaxime, ceftazidime, ticarcillin, and piperacillin-tazobactam (31, 32). In this study, 30 phenotypic patterns were observed. Although each of these 30 isolates were resistant to at least seven antibiotics, one of these strains was resistant to 17 antibiotics, which is a remarkable observation in this study. It is necessary to mention that some of these patterns were comparable with other previously reported patterns in different countries or even Iran. For example, resistance to tobramycin and gentamicin in the current study was similar to the rates reported by previous studies in Iran (35, 36). The emergence

of such MDR and PDR pathogens is a serious crisis in the medical community, an issue that could be solved through development of novel medications.
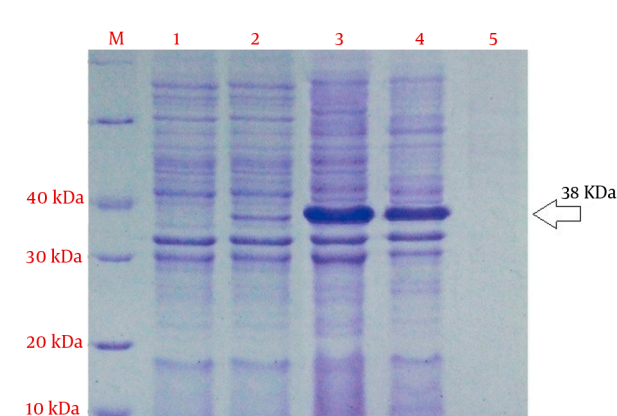
In the next step, *ompA* gene from these resistant strains was amplified using PCR. The *ompA* was considered as antigenic factor for production of the recombinant protein, due to its biological characteristics. The OmpA can attach to the eukaryotic cells, localized in the nucleus and promote apoptosis (7, 37). The OmpA can interact with complement factor H and inhibit immune responses (1). The amplified *ompA* was cloned and sub-cloned in pTZ57RT and in pET-32a(+) expression vector, respectively, and transferred to the *E. coli* host to produce rOmpA. The expressed recombinant OmpA was validated using SDS-PAGE analysis. This study is valuable as the recombinant OmpA protein was ex-
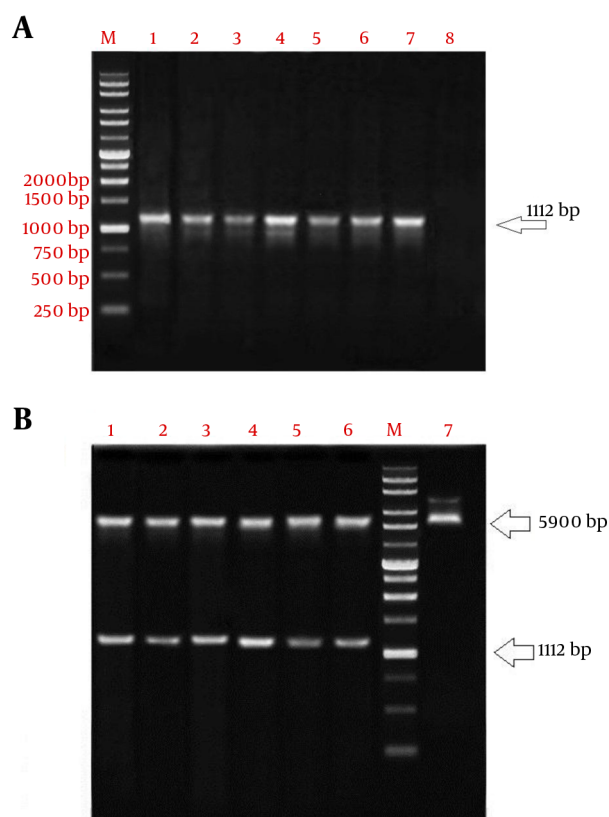
Figure 3. Cloning of *ompA* in pET32a; colony PCR of *ompA*. A, lane M, 1 Kb DNA marker; lane 1 - 7, an amplification of complete *ompA* gene (1112 bp) from pET32a-*ompA*; lane 8, negative control. B, analysis of recombinant expression vector (pET32a-*ompA*) digested by two restrictive endonucleases HindIII and XhoI. Lane 1 - 6, 5900 bp segment pET32a and fragment 1112 bp *ompA*; lane M, 1 Kb DNA marker; lane 7, pET32a undigested.

Figure 4. SDS-PAGE analysis of *A. baumannii* OmpA. SDS-PAGE was used to analyze the expression of rOmpA. Recombinant vectors pET32a-*ompA* and pET32a (+) were transformed to E. coli Rosetta-gami™ 2 (DE3) and induced by 0.9 mM IPTG for 4 h at 37°C. All the samples were analyzed by SDS-PAGE, and the protein was stained with Coomassie Blue in the gel. Lane M, molecular mass marker in 10 kDa; lane 1, whole bacterial lysate of the E. coli Rosetta-gami containing empty pET32a; lane 2, non-induced E.coli Rosetta-gami including pET32a-*ompA*; lane 3 - 4, the whole bacterial lysate and supernatant of the E.coli Rosetta-gami™ 2 (DE3) contained recombinant pET32a-*ompA* which was induced; lane 5, Blank control.
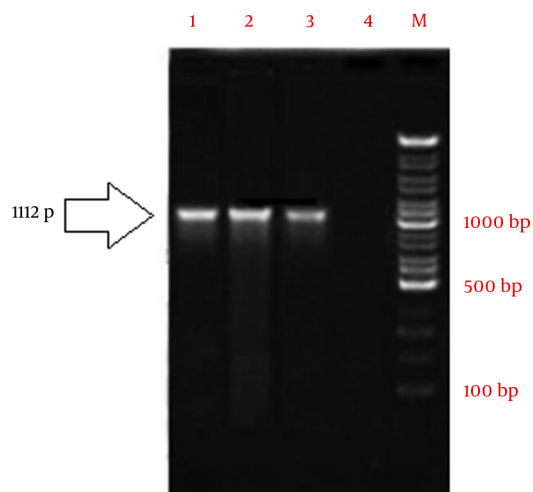
Figure 5. Amplification of *ompA* transcript through the RT-PCR. Lane 1 - 3 shows the amplified cDNA in RT-PCR using specific *ompA* primers. Lane 4 is negative control of the reaction. The lane M illustrates the100 bp DNA size marker.

tracted from the resistant *A. baumannii*, found in the collected samples and not from commercially available standard strains. In a recent publication by Moriel et al., outer membrane proteins *ompA* and *smpA*, were reported as two of 62 potential candidates to develop recombinant vaccine against *A. baumannii* (19).

Additionally, OmpA, showed more than 99% similarity between all *A. baumannii* species, thus, it is a good candidate for vaccine development (38). In 2018, Ansari et al. produced a recombinant construct containing the *ompA* gene, which is notably expressed in HDF eukaryotic cells, and introduced this construct as a promising DNA vaccine candidate (2). Previously, Alzubaidi and Alkozai evaluated the potential of recombinant protein OmpA to study the cellular and humoral responses and found that rOmpA protects the rates against an infectious *A. baumannii* (11). Recently, a recombinant construct was developed to express the recombinant SmpA protein in prokaryotic cells. The

SmpA is one the most important proteins for the localization of outer membrane proteins in the membrane (39). Although limited studies considered ompA as a recombinant vaccine, in the same approach, inactivated whole cell and outer membrane vesicles (OMVs) have been considered as target of vaccine generation against *A. baumanii* (40). As an example, recombinant proteins of Ata, Bap, and the K1 polysaccharide capsular have been produced and recog-

nized as potential immunogens to conflict against *A. baumannii* infections (41-43).

Although the current researchers efficiently expressed the rOmpA in the prokaryotic system, there is a long way to achieve the recombinant protein vaccine. This includes extraction and purification of produced recombinant protein and studying its antigenic potential following injection in the mice experimental model. This is an issue, which should be addressed in the next experiments.

5.1. Conclusions

In this study, the antibiotic resistant feature of *A. baumannii* was revealed in 68 urinary tract infections, wound pus, septicemia, and septum. Among 30 isolates, 29 isolates were defined as MDR and one isolate was PDR. This findings showed the highly resistant pattern of *A. baumannii* in clinical samples. Consequently, the *ompA* gene from resistant isolates was extracted and cloned in a prokaryotic expression vector pET32a(+) to produce the recombinant OmpA protein. It has been observed that the recombinant OmpA protein was highly expressed in *E. coli* at both levels of transcript and encoded protein. The rOmpA may be a promising candidate for vaccine development, although further experiments are urgent. These include purification of recombinant rOmpA protein as well as immunological response assays in mice experimental model. The mentioned issues should be addressed in the near future. To the best of the author's knowledge, this is the first report, in which the *ompA* gene was extracted from resistant *A. baumannii* isolates and cloned in expression plasmid to develop a recombinant protein, which can probably be used as vaccine in the near future, although further experiments are needed.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

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Footnotes

Authors' Contribution: Experimental work and data collection: Hossein Ansari; experimental facilities: Mahdi Bijanzadeh; manuscript writing and data analysis: Maryam Tahmasebi Birgani.

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