

Frequency of *bap* and *cpaA* virulence genes in drug resistant clinical isolates of *Acinetobacter baumannii* and their role in biofilm formation

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ABSTRACT

Objective(s): *Acinetobacter baumannii* has a high propensity to form biofilm and frequently causes medical device-related infections with multiple-drug-resistance in hospitals. The aim of this work is to study antimicrobial resistance and the role of *bap* and *cpaA* genes in biofilm formation by *A. baumannii* to understand how this pathogen persists in the hospital environment.

Materials and Methods: The antibiotic resistance profile and *in vitro* biofilm-forming ability of one hundred clinical isolates of *A. baumannii* was evaluated by disc diffusion and crystal-violet staining methods, respectively. Isolates were tested for the presence of *bap* and *cpaA* genes.

Results: The isolates were highly resistant to cefepime, third-generation cephalosporins, ciprofloxacin, cotrimoxazole, aminoglycosides and carbapenems. Moreover, four isolates were resistant to colistin. Quantification of biofilm showed that 43% of the isolates were strong biofilm-producer. Furthermore, 32% of the isolates exhibited moderate biofilm-formation and showed initial binding activity. Frequency of *bap* and *cpaA* were determined 92% and 36%, respectively.

Conclusion: There was strong association between the presence of *bap* gene and biofilm formation by *A. baumannii* isolates ($P=0.003$). In addition, multidrug resistant isolates produced stronger biofilm than other isolates ($P=0.0001$). These results indicate importance of biofilm in resistance of isolates and effect of presence of *bap* gene in biofilm formation by *A. baumannii* strains.

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Introduction

The growing prevalence of multi- or pan-drug resistant *Acinetobacter baumannii* strains has resulted in limited treatment choices. Most of *A. baumannii* strains are resistant to clinically valuable antibiotics (1), with inferable deaths up to 43% for patients under intensive care and 23% for hospitalized patients (2). In fact, biofilm production is another effective route for bacteria to survive in the presence of antibiotics (3), particularly for *A. baumannii* that causes biofilm-related contamination of medical devices (4).

Given the implications that biofilm formation has in medical care, isolation of bacterial species from biofilms and detection of the genetic determinants to control this complex procedure became an active field of research over the last few years. Biofilms are a dynamic, highly structured heterogeneous communities of bacteria inside a polymeric conglomerate

of polysaccharides and proteins that have integrated metabolic activities and create sessile phenotypes markedly different from their planktonic partners (5, 6). Bacterial biofilms have been found on surfaces of plastic, glass and numerous sorts of medical devices as well as on a kind of other surfaces in the hospital environment (7). Biofilms are resistant to antibiotics, detergents and host immune defenses, and antibiotic resistance of microorganisms in these colonies can be increased up to 1000-fold (3).

As in different pathogens, a substantial protein expressed at the *A. baumannii* cell surface, known as biofilm-associated protein (Bap), has a role in biofilm production as in host cell adherence (8). Bap family members are characterized as high-molecular weight proteins that are available on the bacterial surface, contain a core domain of tandem repeats, and give to bacteria the ability to form a biofilm (9). Since

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the initial identification of Bap, its homologue counterparts have been recognized in at least 13 pathogenic species, and the proteins commonly share functional and structural similarities, although not necessarily primary sequence similarity (9, 10). Bap production has been linked to primary adherence to abiotic surfaces, biofilm formation in both Gram-positive and Gram-negative bacteria, persistence and subsequent pathogenesis (11). The *A. baumannii* Bap protein is important for stabilization of mature biofilms on glass, influencing both biovolume and thickness (12). This information recommends that *A. baumannii* Bap is a key factor in biofilm production and therefore may have a role in persistence in the hospital environment and in infection.

A. baumannii bacteremia regularly originates in the respiratory tract and moves to the circulatory system where it is associated with very high mortality rates due at least in part to disseminated intravascular coagulation (DIC) or septic shock (13). Patients suffering from *A. baumannii* infections have been appeared to have unusual coagulation-related proteins (14), which are basic to antimicrobial defense (15, 16) and a target of various Gram-negative virulence factors (17). Here, we investigate CpaA (coagulation targeting metallo-endopeptidase of *A. baumannii*), a novel reprotolysin-like secreted metallo-endopeptidase, present in clinical isolates. To our knowledge, few studies have examined association between *cpaA* gene and the coagulation as a means of *A. baumannii* virulence. Therefore, we chose to study biofilm formation by *A. baumannii* and the prevalence of *bap* and *cpaA* genes in order to understand how this pathogen persists in the hospital environment to cause outbreaks worldwide.

Materials and Methods

Sample collection

A total of 100 *A. baumannii* isolates from different clinical sources were collected from three referral University affiliated health-care centers (Imam Reza, Sina, and Children) in Tabriz, Iran during May 2015 to July 2016 (14 month time period). These isolates were isolated from urine, wound, blood, bronchial secretion, pharynx, bile and tracheal aspirates. All presumptive *Acinetobacter* spp. isolates, which were Gram-negative diplococci, non-lactose fermentative and oxidase-negative, were identified as *A. baumannii* by utilizing the conventional methods and growth potential at 37 °C and 44 °C (18). Later, polymerase chain reaction (PCR) amplification was performed to confirm species identification using the intrinsic *bla_{oxa-51}*-like allele as described previously (19). Finally, genomic DNA of the isolates was extracted as described previously (20).

Ethics standards

Ethics approval to perform the study was obtained from the institutional review board of

Tabriz University of Medical Sciences. Written informed consent was obtained from all patients included in the study.

Antimicrobial susceptibility testing

Kirby-Bauer disk diffusion methods was performed to determine antimicrobial susceptibility patterns in accordance with the recommendations of the clinical and laboratory standards institute (CLSI) (21). The tested antimicrobial agents that were purchased from Hi-media, India were as follow: ciprofloxacin (30 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), cotrimoxazole (25 µg), amikacin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), amikacin (30 µg), cefepime (30 µg), tetracycline (10 µg), and colistin (10 µg). *Escherichia coli* ATCC 25922 was used as a quality control strain. Multidrug resistance (MDR) was characterized as resistance of an isolate to three or more unrelated antimicrobial agents (22). Resistance to one drug class was regarded as resistance to all antimicrobial members of that class.

Biofilm formation assay

A. baumannii biofilm formation was analyzed in 96-well flat bottom polystyrene plates, under static conditions for 48 hr as previously described (23, 24). For biofilm development, inoculums of around 10⁷ CFU/ml were prepared by adjusting culture grown bacterial suspensions in trypticase soy broth (TSB) (Hi-media, India) from overnight cultures to an optical density (OD_{600 nm}) of 0.1 and adding 100 µl of every inoculums to wells. After 48 hr incubation, plates were tenderly washed one time with 1X phosphate buffered saline (PBS; pH 7.4) and stained with 100 µl of 0.1% Crystal Violet (CV) for 30 min at room temperature. Excess CV was expelled by washing, and biofilm was evaluated by measuring absorbance of the supernatant at 570 nm utilizing a microtiter plate reader after the solubilization of CV in 95% ethanol. Biofilm assays were performed in triplicate for each clinical strain and the mean biofilm absorbance quality was determined.

bap and cpaA gene detection

The specific primers for the *bap* gene (Bap-F, 5' TAG GGA GGG TAC CAA TGC AG and Bap-R, 5' TCA TGA TTT GAT GCT GCA GCG ATA A) and the *cpaA* gene (CpaA-F, 5' CTG CTT TAG GAA AAT GGG and CpaA-R, 5' CGC CTT CAA TCA TTC TAA G) were used to evaluate the biofilm formation (17, 25). The *bap* gene was amplified under the following conditions: 95 °C for two min, 95 °C for 30 sec, 61 °C for 30 sec, 68 °C for one min (30 cycles), and 68 °C for two min. Also, PCR condition for *cpaA* gene was as follow: 95 °C for three min, 95 °C for 30 sec, 63 °C for 30 sec, 72 °C for one min (35 cycles), and 68 °C for five min. The PCR products were separate in 1.5% agarose gel.

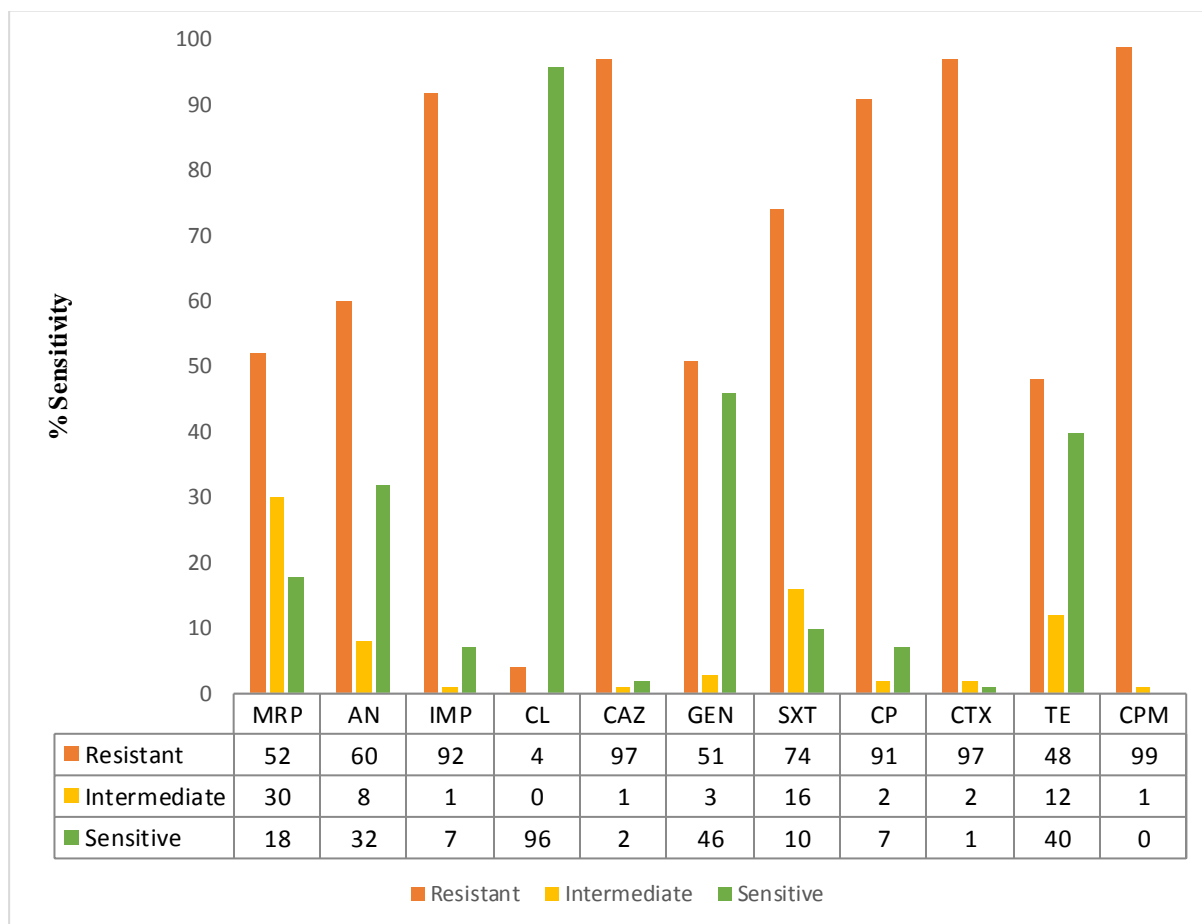


Figure 1. Antibiotic susceptibility of the *Acinetobacter baumannii* isolates. Abbreviations; MRP, meropenem; AN, amikacin; IMP, imipenem; CL, colistin; CAZ, ceftazidime; GEN, gentamicin; SXT, cotrimoxazole; CP, ciprofloxacin; CTX, cefotaxime; TE, tetracycline; CPM, ceftazidime. Numbers depicted in the above figure present susceptibility as percentage of bacterial isolates. Mueller-Hinton agar was used for susceptibility test. The inoculum concentration was 1.5×10^8

Statistical methods

All statistics have been evaluated by means of SPSS software version 22.0 (SPSS Inc, Chicago, IL, USA). Comparative data was calculated utilizing the Fisher’s exact and two-tailed χ^2 test, once appropriate. A *P*-value of ≤ 0.05 was thought to be significant.

Results

Descriptive epidemiology

In the present study, 100 non-duplicative *A. baumannii* was isolated from different clinical specimens, including tracheal aspirates (50%), wound (15%), urine (13%), blood (12%), pharynx (5%), bronchial secretion (4%), and bile aspirates (1%). The *A. baumannii* isolates were collected from different wards of the three referral and University affiliated hospitals includes Imam Reza (51%), Sina (38%), and Children (11%) centers. The most clinical isolates were obtained from intensive care units (56%) and other isolates were obtained from other wards such as, burn, lung, internal, surgery, kidney, neurology and trauma, respectively. The mean age of the patients was 49 ± 24 years and the ratio between genders was 1:0.8 (males/females). Of the isolates identified as *Acinetobacter* spp.

phenotypically, all confirmed as *A. baumannii* by using OXA-51-like primers and observing ~ 353 bp band in the agarose gel.

Antimicrobial susceptibility testing

Resistance to ceftazidime was the most common (99%), followed by cefotaxime and ceftazidime (97%), imipenem (92%), ciprofloxacin (91%), cotrimoxazole (74%), amikacin (60%), meropenem (52%), gentamicin (51%), tetracycline (48%), and colistin (4%) (Figure 1). Antibiotic susceptibility profiles of *A. baumannii* isolates according to the different specimen from the hospitalized patients were shown in Table 1. About 90% of the isolates were resistant to at least one of the tested carbapenems antibiotics, which included imipenem and meropenem. No isolate was resistant to all of the 11 antibiotics. Of the 100 *A. baumannii* isolates tested, all were resistant to at least one antibiotic. Additionally, 88 isolates were classified as MDR.

Biofilm formation

Evaluation of biofilm formation by microtiter plate method presented 43% (n=43) of the isolates

Table 1. Antibiotic resistance patterns of *Acinetobacter baumannii* isolates according to various specimens

Specimen	CP	IMP	MRP	GEN	SXT	AN	CAZ	CTX	CPM	TE	CL
Tracheal aspirates	45	46	23	22	39	26	48	49	49	23	3
Urine	12	11	5	5	10	8	13	13	14	6	0
Wound	14	13	10	8	10	8	14	14	14	7	0
Blood	10	12	8	7	8	9	12	11	12	7	1
Bronchial secretion	4	4	2	3	2	3	4	4	4	1	0
Pharynx	5	5	3	5	4	5	5	5	5	3	0
Bile	1	1	1	1	1	1	1	1	1	1	0
Total	91	92	52	51	74	60	97	97	99	47	4

MRP: meropenem; AN: amikacin; IMP, imipenem; CL, colistin; CAZ, ceftazidime; GEN, gentamicin; SXT, cotrimoxazole; CP, ciprofloxacin; CTX, cefotaxime; TE, tetracycline; CPM, cefepime

as strong biofilm producer; moreover, these isolates had stronger initial attachment capacity to microtiter wells. About 32% (n=32) displayed moderate biofilm formation, which indicated moderate initial binding activity, while weak or no biofilm was observed in 25% (n=25) of the isolates.

Presence of bap and cpaA genes

All isolates involved in this study possessed one of the *A. baumannii* bap and/or the cpaA genes.

Ninety-two (92%) isolates in the study possessed the *A. baumannii* bap gene and 36 (36%) possessed the cpaA gene. Of the isolates that had bap, 64 possessed it alone and the other 28 ones had the combination of bap and cpaA. The percentage of virulence gene detected in *A. baumannii* strains according to the site of isolation was shown in Table 2. Furthermore, the relation between presence of each virulence genes and biofilm formation are presented in Table 3.

Table 2. The percentage of virulence genes detected in *Acinetobacter baumannii* strains according to the site of isolation

Genes		Tracheal aspirates	Urine	Wound	Blood	Bronchial secretion	Pharynx	Bile	Total
bap	Positive	45	12	13	11	5	5	1	92
	Negative	5	1	1	1	0	0	0	8
cpaA	Positive	16	5	3	6	5	1	0	36
	Negative	34	8	11	6	0	4	1	64
Total		50	13	14	12	5	5	1	100

Table 3. Statistical analysis of the relationship between biofilm formation and the presence of bap and cpaA genes in *Acinetobacter baumannii* strains with multidrug resistance (MDR)

		bap		cpaA		MDR		Total
		Positive	Negative	Positive	Negative	Positive	Negative	
Biofilm	Weak	19 (76.0%)	6 (24.0%)	4 (16.0%)	21 (84.0%)	16 (64.0%)	9 (36.0%)	25
	Moderate	32 (100%)	0 (0%)	12 (37.5%)	20 (62.5%)	31 (96.9%)	1 (3.1%)	32
	Strong	41 (95.5%)	2 (4.0%)	20 (46.5%)	23 (53.5%)	41 (95.3%)	2 (4.7%)	43
Total		92 (92.0%)	12 (12.0%)	36 (36.0%)	64 (64.0%)	88 (88.0%)	12 (12.0%)	100
P-value		0.003		0.40		0.000		

Discussion

A. baumannii is an important nosocomial pathogen that is omnipresent in environment, associated with MDR and hospital outbreaks of infection resulting in high levels of morbidity and mortality, especially in the intensive care units (26, 27). It also accounts for almost 80% of all reported *Acinetobacter* infections, including bacteremia, urinary tract infections, ventilator-associated pneumonia, peritonitis, wound infections and meningitis (26). These infections have turned into a challenge for clinicians and a real threat to international public health because of the rapid spread of antimicrobial resistance and the slow development of novel antimicrobials (28). Moreover, the genetic capability of

MDR *A. baumannii* to carry and transfer diverse antibiotic resistance determinants represents a noteworthy risk in hospitals. This contributes largely to the lethality and severity of infections, in addition to the high rate of chronic infections, as the organism is able to persist and survive for a long period of time, even after treatment with antibiotics and disinfectants.

In the recent years, the antimicrobial susceptibility rates have been highly decreased among *A. baumannii* isolates in Iran; there are generally more resistance to antibiotics in Iran than those from different regions of the world (29, 30). The current study found 100 isolates of *A. baumannii* collected from different wards of three main hospitals in Tabriz, Iran, which were highly resistant to different

classes of antibiotics, and the prevalence of MDR isolates was 88%. The rate of carbapenems resistance in *A. baumannii* is also increasing during the past years in our region, and in this study, we found that up to 92% and 60% of isolates were resistant to imipenem and amikacin, respectively. However, the profile of antibiotic resistance shows with the exception of carbapenems, amikacin was relatively the most active antimicrobial agent against most isolates of *A. baumannii* (with susceptibility rate of 41.5%) (30). According to the data, resistance to colistin in non-fermenters such as *A. baumannii* is relatively low (28). In our study, 4% of isolates were resistant to this drug. While, in other studies from the North and Northwest of Iran, the rate of 16% and 19% have been reported, respectively (31, 32).

A. baumannii and *Pseudomonas aeruginosa* are the most common bacteria that form biofilm. In our study, 75% of these *A. baumannii* clinical isolates exhibited a higher capacity for biofilm formation, and multidrug resistant isolates produced stronger biofilm than other isolates ($P=0.000$) (Table 3). This attachments as biofilm in microtiter plates contributed to their outstanding antibiotic resistance properties to a wide variety of antibiotics (33). This is a relatively higher rate than that observed in other studies. However, evaluation of biofilm formation by microtiter plate method in another study from Iran showed that among 81% of carbapenem non-susceptible *A. baumannii*, 52% of the isolates were biofilm producers (34). In Korea, it was reported that all 23 (100%) strains of MDR *A. baumannii* formed biofilm (35). The results of studies from Spain and the United States showed that 63% (36) and 55% of *A. baumannii* strains isolated from wounds were biofilm-forming strains, with a median biofilm mass of 0.125 ± 0.061 (37).

Yet, presence and expression of genes associated with biofilm formation ought to be confirmed by genotypic characterization methods. Bap were initially characterized in *Staphylococcus aureus* (38) and has since been recognized in a number of other Gram-negative and Gram-positive pathogenic bacteria. They are characterized by functional qualities and shared structural features and are essentially high-molecular-weight, repetitive surface proteins involved in biofilm formation (39). To determine whether Bap expression is involved in biofilm formation on medically relevant surfaces, biofilm studies were performed on polystyrene,

polypropylene, and titanium. As shown in Table 3, there was strong association between the presence of *bap* gene and biofilm formation by *A. baumannii* isolates ($P=0.003$) in this study. A recent study has shown that Bap plays a role in static biofilm maturation and maintenance, increasing both biofilm thickness and biovolume on glass surfaces (12). Moreover, in a study that conducted in Iran, the

frequency of *bap* gene related to biofilm formation was 30% (40). However, in our study and another study from Australia (from a single outbreak), 92% of *A. baumannii* isolates possess this gene (8, 41). As we determined using the CV staining technique, *A. baumannii* isolates carrying the *bap* gene and those without *bap* vary significantly in their biofilm formation capabilities ($P<0.05$) (Table 3). The biofilm data presented in this study along with other studies confirm that Bap is an extremely important factor in biofilm development and this surface protein is needed in the formation of a classic biofilm phenotype on surfaces. These results are important because the ability of *A. baumannii* to survive and persist on abiotic materials is linked to outbreaks of nosocomial infections. In order to develop more effective ways of eradicating *A. baumannii* contamination from the environment, we must identify the bacterial factors that are important for persistence, and our data implicate Bap as a key component.

Various bacterial secreted proteases are known to interact with host systems to promote infection. According to the data, CpaA-treated plasma has reduced clotting activity in contact pathway-activated partial thromboplastin time (aPTT) assays, but increased clotting activity in tissue factor pathway prothrombin time (PT) assays (17). CpaA has limited homology to any other described peptides in regions other than the active site; it was therefore important to determine whether the protease is active, and whether it targets host-derived substrates as a potential virulence mechanism. In our study, we detected the secreted protease CpaA of 36 *A. baumannii* clinical isolate (Table 3); while, in other research, 12/16 (75%) of *A. baumannii* clinical isolates generated amplicons from primers designed to amplify *cpaA* gene (17).

Conclusion

To the best of our knowledge, this is the first research to examine the association between *cpaA* gene and the coagulation as a means of *A. baumannii* virulence in Iran. This study indicates that the presence of the *cpaA* gene among *A. baumannii* isolates is not associated with *in vitro* formation of biofilm ($P>0.05$). However, there was strong association between presence of *bap* gene and biofilm formation by *A. baumannii* isolates ($P=0.003$). In addition, multidrug resistant isolates produced stronger biofilm than other isolates ($P=0.000$). These results indicate importance of biofilm in resistance of isolates and effect of the presence of *bap* gene in biofilm formation by *A. baumannii* strains. Moreover, these results recommend the need for further investigation involving a large number of multicentric *A. baumannii* isolates collected from Iran.

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Conflicts of interest

The authors declare that no conflict of interest exists.

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