Virulence determinants and biofilm formation of *Acinetobacter baumannii* isolated from hospitalized patients

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Abstract

Introduction *Acinetobacter baumannii* are nosocomial bacteria that are responsible for outbreaks and severe infections in hospitalized patients globally. The major target of this study was the characterization of virulence determinants and biofilm formation of *A. baumannii* isolates from hospitalized patients.

Methods In total, 100 *A. baumannii* were collected from three hospitals in Tehran, Iran, 2017-2018. The isolates were assessed using phenotypic and genotypic methods and then screened for virulence factor encoding genes such as *plcN* and *lasB* using conventional polymerase chain reaction. Furthermore, bacterial biofilm formation, motility and hemolytic and proteolytic activities were assessed.

Results Of 100 *A. baumannii* isolates, 20 isolates included *plcN* and four isolates included *lasB* using PCR assay. Overall, 21 isolates were negative for biofilm formation while 45, 20 and 14 of the total isolates were reported as weak, moderate and strong biofilm producers, respectively. All isolates were positive for *bap* genes using PCR. Moreover, 35 isolates were motile on Luria-Bertani media, 47 isolates were α -hemolytic on Brucella blood agar media and all isolates displayed proteolytic activity.

Conclusions Healthcare-associated infections with *A. baumannii* are a major concern, importantly due to their potency to acquire virulence factor genes. Therefore, shedding light in the discovery of new antimicrobial and/or therapeutic agents against virulent *A. baumannii* strains seem to be necessary.

Keywords *Acinetobacter baumannii*, biofilm formation, virulence factors, *lasB*, *plcN*.

Introduction

Acinetobacter baumannii is a nosocomial pathogen that produces a variety of infections in humans, including endocarditis, meningitis, pneumonia (in mechanically ventilated patients), septicemia and urinary tract infection (UTI).¹ Since *A. baumannii* can persist on hospital surfaces and equipment and emerge as a multidrug resistant (MDR) pathogen, infections by this bacterium are a major concern for clinicians as well as medical researchers.² Up to date, a small number of virulence factors have been reported

for *A. baumannii*, compared to virulence factors reported for other Gram-negative bacteria. These virulence factors, including efflux pumps, hemolytic factors, iron acquisition systems, lipopolysaccharides and OmpA, can induce host immune system responses or bacterial adherence to epithelial cells.³ Previous studies have reported that phospholipases C (PLCs) and D are the major phospholipase classes in *A. baumannii*, which hydrolyze phospholipids and hence facilitate infection spreads in hosts. Elastase is another major virulence factor that causes degradation of elastin and destruction of the host tissues or

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defense mechanisms.^{4,5} The two enzymes phospholipase and elastase were previously reported for *Pseudomonas aeruginosa* and demonstrated a critical role in mammalian infections.⁵ Production of biofilms by Bap protein and *csu* locus (encoding the chaperone-usher *Csu*) is another essential characteristic of *A. baumannii* that induces pathogenicity and is associated with the bacterial extended survival in hospitals.^{6,7} Deletion or dysfunction of the virulence factor associated genes may decrease in vivo virulence of *A. baumannii*. Furthermore, virulence factors are responsible for adhesion, colonization and invasion of *A. baumannii* strains.^{2,8} Therefore, the major target of the current study was assessment of phospholipase (*plc*) and elastase (*las*) genes, biofilm formation, motility and hemolytic and proteolytic activities in *A. baumannii* isolates from hospitalized patients.

Methods

Setting

In this study, a total of 100 clinical *A. baumannii* isolates were collected from various wards of three tertiary referral hospitals abbreviated as H1, H2 and H3 in Tehran, Iran, 2017-2018. These hospitals have at most 1000, 453 and 250 active beds, respectively. H1 is a major tertiary referral hospital that admits patients from other hospitals or clinics throughout Iran and it also provides the primary care for local patients. H2 and H3 are teaching hospitals affiliated with a medical university and operate as primary and tertiary care centers. All isolates were characterized using phenotypic and genotypic methods such as morphology, API 20 NE systems (bioMérieux, Marcy-l'Étoile, France) and polymerase chain reaction (PCR) (Peqlab Biotechnologie GmbH, Erlangen, Germany) of intrinsic carbapenemase *bla*_{oxa-51-like} gene (Table 1).⁹ The *A. baumannii* ATCC 19606 was used as reference in all assessments.

Molecular detection of phospholipase (*plcN*) and elastase (*lasB*) genes

Genomic DNA extraction was carried out using standard phenol-chloroform extraction method.¹⁰ The phospholipase (*plcN*) and elastase

(*lasB*) genes were detected using specific primers (Table 1).¹¹ The PCR conditions included an initial denaturation step of 95°C for 10 min; followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. The final elongation step was set at 72°C for 10 min. The predicted amplicon sizes of *plcN* and *lasB* genes included 466 and 300 bp, respectively. The *P. aeruginosa* strain PAO1 was used as positive control in PCR amplification of *plcN* and *lasB* genes.

Biofilm formation assay

The biofilm formation capability of *A. baumannii* was investigated using the microtiter-plate assay.¹² After preparation of bacterial suspensions in trypticase soy broth (TSB) (Merck, Darmstadt, Germany) with an OD₆₀₀ of 0.1, suspensions were inoculated in 96-well polystyrene microplates and incubated at 37°C for 24 h. Microplates were washed thrice with 1× phosphate buffered saline (PBS). These were then fixed using absolute methanol and stained using 1% crystal violet. The optical absorbance was recorded at 570 nm and results were analyzed based on the criteria described previously.¹² Furthermore, N-terminal (5') regions of biofilm associated protein (*bap*) genes were amplified using specific primers (Table 1).¹³

Motility assay

For detection of surface motility, one colony of *A. baumannii* was grown in Luria-Bertani (LB) (Merck) broth at 37°C for 24 h. The bacterial suspension turbidity was adjusted to 0.5 McFarland (10⁸ CFU/mL) and then 10 µL of the suspension were cultured on LB agar plates of 0.3%. These plates were incubated at 37°C for 24 h and the growth area (cm²) was calculated.¹⁴ The bacterial motility was categorized based on the criteria described previously. These criteria included (i) negative (-) if the motility zone was <5 mm; (ii) intermediate (+) if the motility zone was 5-20 mm; and (iii) highly motile (++) if the motility zone was >20 mm.¹⁵

Hemolytic activity

To identify hemolytic activity, a single colony from *A. baumannii* was dissolved in LB broth

Table 1. Primers used in this study for the PCR amplification of target genes

Target gene	Primer sequence	Size (bp)*
<i>bla</i> _{oxa-51-like}	5'-TAATGCTTTGATCGGCCTTG-3' 5'-TGGATTGCACCTTCATCTTGG-3'	353
<i>bap</i>	5'-ATGCCTGAGATACAAATTATTGCCAAGGATAATC-3' 5'-AGGTGCTGAAGAATCATCATCATTAC-3'	560
<i>plcN</i>	5'-GTTATCGCAACCAGCCCTAC-3' 5'-AGGTGCAACACCTGGAACAC-3'	466
<i>lasB</i>	5'-GGAATGAACGAAGCGTTCTC-3' 5'-GGTCCAGTAGTAGCGGTTGG-3'	300

*Amplicon size

(Merck) and incubated at 37°C for 24 h. This suspension was adjusted to 0.5 McFarland and 10 µL of the suspension were cultured on 5% Brucella blood agar (BBA) (Merck) plates. These were then incubated at 37°C for one week.¹⁶

Proteolytic activity

The proteolytic activity of *A. baumannii* was assessed based on previous reports. Briefly, one bacterial colony was suspended in TSB and incubated at 37°C for 24 h with agitation. The bacterial suspension (turbidity of 0.5 McFarland) was centrifuged and the supernatant was filter (0.22 µ) sterilized. Then, 0.5 mL of the bacterial supernatant were incubated with 0.5 mL of azoalbumin solution (1 mg/mL) (Merck) at 37°C for 24 h with agitation. After incubation, trichloroacetic acid (13%) (Merck) was added to tubes and incubated at -20°C for 30 min. The procedure was followed by centrifugation and OD₄₄₀ measurement of the bacterial supernatants.¹⁷

Data analysis

Descriptive statistics were performed using SPSS Software v.18 (SPSS Inc., Chicago, IL, USA).

Results

Overall, 100 *A. baumannii* isolates were recovered from the patients (55% males and 45% females) admitted to hospitals H1 (41%), H2 (36%) and H3 (23%), aged 1-60 years old. Out of the total isolates, 35% were recovered from neonatal, 30% from infectious disease, 20% from intensive care unit (ICU), 10% from emergency and 5% from surgery wards. Furthermore, 89%

of the isolates were from wounds, 6% from blood and 5% from catheters.

Twenty isolates included *plcN* and four isolates included *lasB*. Six *plcN* positive *A. baumannii* (30%) were isolated from hospital H1 and other nine (45%) and five (25%) isolates from hospitals H2 and H3, respectively. One *A. baumannii* isolate (25%) harboring *lasB* was isolated from hospital H1, one (25%) from hospital H2 and two (50%) from hospital H3.

In the biofilm formation assay, OD₅₇₀ values (mean ± standard deviation) of the negative control included 0.012±0.014. These values ranged between 0.013-0.024 in 21 isolates interpreted as negative (-), 0.05-0.06 in 45 isolates interpreted as weak (+), 0.09-0.13 in 20 isolates interpreted as moderate (++) and 0.17-0.24 in 14 isolates interpreted as strong (+++) biofilm producers. Of *plcN* and *lasB* positive isolates, eight (40%) were weak, seven (35%) moderate and five (25%) strong biofilm producers.

The PCR amplification of *bap* N-terminal (5') regions was positive for all isolates (Table 2). Moreover, 35 isolates were motile on LB agar after 24 h of incubation (motility diameters ranged from 30.33±3.62 to 10.28±2.93 mm). All *plcN* and *lasB* positive isolates showed strong motility in LB agar. Hemolytic activity tests showed that 47 *A. baumannii* isolates were α-hemolytic in BBA media. Of *plcN* and *lasB* positive isolates, 14 were positive for α-hemolysis as well after 24 h of incubation. Results demonstrated that proteolytic activity of the isolates ranged from 40.90±6.7 to 12.21±5 U/L (Table 2).

Table 2. Results for the virulence factor assessment of *Acinetobacter baumannii* isolates

Isolate	<i>plcN</i> gene	<i>lasB</i> gene	Biofilm formation*	<i>bap</i> gene	Motility assay (mm)**	Hemolytic activity	Proteolytic activity (U/L)**
AB1	+	-	+	+	28.43 ± 2.62	-	12.21 ± 5.0
AB2	+	-	+	+	20.38 ± 3.40	+	30.39 ± 9.6
AB3	+	-	++	+	20.1 ± 6.82	-	31.28 ± 1.5
AB4	+	-	+	+	20.38 ± 7.92	-	31.58 ± 19.1
AB5	+	-	+	+	23.46 ± 2.12	+	24.13 ± 4.9
AB6	+	+	++	+	33.6 ± 5.52	+	40.90 ± 6.7
AB7	+	+	+	+	28.69 ± 5.41	+	29.49 ± 4.4
AB8	+	-	+	+	30.33 ± 3.62	+	23.24 ± 0.8
AB9	+	-	+++	+	20.55 ± 1.20	+	21.15 ± 1.8
AB10	+	-	++	+	20.18 ± 2.12	-	29.20 ± 7.7
AB11	+	+	++	+	25.6 ± 7.92	+	37.67 ± 13.6
AB12	+	-	++	+	25.3 ± 1.60	-	28.0 ± 7.6
AB13	+	-	+++	+	20.3 ± 4.83	+	36.3 ± 7.2
AB14	+	-	+++	+	21.5 ± 5.41	-	23.54 ± 2.0
AB15	+	-	+	+	20.1 ± 5.66	+	37.8 ± 1.8
AB16	+	-	++	+	24.21 ± 5.68	+	31.88 ± 10.6
AB17	+	+	++	+	26.2 ± 3.30	+	28.0 ± 2.7
AB18	+	-	+++	+	20.56 ± 7.30	+	32.9 ± 7.7
AB19	+	-	+++	+	20.13 ± 5.77	+	13.7 ± 7.2
AB20	+	-	+	+	20.1 ± 1.22	+	23.24 ± 0.8

*Biofilm production was interpreted as: negative (-), weak (+), moderate (++) strong (+++).

**Data are presented as mean ± standard deviation.

Discussion

In recent years, *A. baumannii* has repeatedly been reported as a nosocomial pathogen that spreads within hospitalized patients. The clinical importance of *A. baumannii* depends on the wide genetic spread of resistance genes and the bacterial persistence in hospitals, especially in intensive care units (ICUs).^{18,19} Additionally, *A. baumannii* includes virulence factors, which help the bacteria in pathogenesis.⁷ Phospholipase is an important enzymatic virulence factor in Gram-negative bacteria, which hydrolyzes phospholipids in mammalian cell membranes).²⁰ Phospholipase activity was basically found in bacterial species (e.g., *P. aeruginosa*) that caused human respiratory tract infections and destroyed respiratory tissues.²¹ The current study demonstrated that nearly 20% of the isolates included *plcN* that could be associated with the bacterial phospholipase activity. In a study by Kareem et al., they found that 23.3% of the Iraqi *A. baumannii* isolates were positive by PCR for *plcN*.¹¹ Doughari et al.

demonstrated a 50% sequence identity between phospholipase enzymes of *A. baumannii* and *P. aeruginosa*.¹⁸ Presence of phospholipases A, C or D in *A. baumannii* could be essential for the improvement of epithelial cell toxicity.⁴ Ali et al. showed that mutation in phospholipase genes decreased the cytotoxic effects of *A. baumannii*.³ In fact, *A. baumannii* produces elastase and can destroy host tissues such as lung and urinary tract tissues.³ The protease activity of elastase was previously reported in *P. aeruginosa* infections, especially in chronic obstructive pulmonary disease.²² In general, the increasing number of *A. baumannii* infections due to the bacterial persistence in hospital environments and acquiring of virulence factors by the bacteria are major health concerns. In the present study, PCR of *lasB* showed that 4% of the *A. baumannii* isolates included elastase genes. A previous study by Kareem et al. demonstrated *lasB* genes in nearly 53.3% of the *A. baumannii* isolates.¹¹ These results were further supported using biofilm formation assay, in which *A. baumannii* isolates with elastase

clearly showed enhanced adhesion and invasion potencies. In the biofilm formation assay, a majority of *A. baumannii* isolates with *plcN* and *lasB* formed biofilm weakly. As a rule, *A. baumannii* can survive for prolonged times in hospitals due to the bacterial excellent ability of producing biofilms on inanimate surfaces.¹⁹ Although all *A. baumannii* isolates included 5'-end of *bap* gene in this study, nearly 21% of them were negative in biofilm formation assay. In addition to *bap* gene, biofilm production capacity depends on additional genes such as pili assembly system (*csu*) and *ompA*.^{19,20} Furthermore, identification of other bacterial virulence determinants (e.g., motility, hemolysis and proteolytic activities) revealed that *plcN* and *lasB* positive isolates were strongly virulent.¹¹ Relatively, virulence determinants of individual *A. baumannii* isolates differed greatly. Although no associations were found between the bacterial virulence determinant factors and the biofilm formation ability, all virulent *A. baumannii* isolates could form biofilms. These virulence factors normally contribute to resistance against immunological defense mechanisms. In the present study, antibiotic susceptibility tests demonstrated that all virulent *A. baumannii* isolates were MDR as well (data not shown). These interesting data suggested that virulent *A. baumannii* isolates could trigger severe diseases. However, data need to be further verified in future studies.

Conclusions

The current study was the first study on *plcN* and *lasB* gene detection in *A. baumannii* isolates in Iran; however, a few reports have been published on other *A. baumannii* virulence determinants in Iran and Middle East. As *A. baumannii* can acquire virulence and resistance genes quickly, further studies on *A. baumannii* virulence factors are necessary to find novel therapeutic agents or vaccines against this nosocomial pathogen.

Authors' contributions statement: AA carried out the literature review, laboratory tests, data analysis and initially drafted the manuscript. MS participated in study design. RMNF contributed to final writing and editing the manuscript. FM designed and supervised the study. All

authors have read and approved the final version of the manuscript.

Conflicts of interest:

All authors – none to declare

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