

Virulence Factor Profile and Biofilm Formation Ability of *Acinetobacter baumannii* Strains Isolated from Hospitalized Patients

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ABSTRACT

Background: *Acinetobacter baumannii* is an opportunistic pathogen which plays crucial roles in nosocomial infections and hospital outbreaks.

Aim: To investigate to determine the presence of virulence factors and the ability of biofilm production by isolated *A. baumannii* from hospitalized patients.

Methods: The prevalence of *A. baumannii* in clinical samples was identified with standard biochemical tests. Polymerase chain reaction (PCR) was used for investigation of the presence of virulence factors in isolated strains.

Results: Isolates were assayed for biofilm formation by microtiter plate method. Of 120 samples, 100 (83.33%) were positive for *A. baumannii*. Although the presence of *iutA* (10%) and *papC* (5%) virulence genes was confirmed by PCR, isolated *A. baumannii* were negative for *cnf1*, *cnf2*, *fnb*, and *kpsMT II* virulence factors. Moreover, the quantification of biofilm production showed that 61% of the isolates were able to produce biofilm ($P=0.028$). Also, there was no relationship between the presence of *iutA* and *papC* genes and biofilm formation. The significant rates of *A. baumannii* isolates were able to form biofilm which could lead to their survival in a hospital environment.

Conclusion: Iron uptake and fimbriae could be considered as important virulence factors in survival and adherence of *A. baumannii*.

Keyword: *Acinetobacter baumannii*, Polymerase chain reaction, Biofilm, *iutA*, *papC*

INTRODUCTION

One of the most causes of mortality and morbidity worldwide is hospital-associated infections (HAIs). Among the reasons for HAIs, bacteria play an important role, and *Acinetobacter baumannii* is one of the most predominant bacteria leading to HAIs¹. *A. baumannii* is an opportunistic pathogen which causes a wide range of infections including pneumonia², bacteremia³, urinary tract infections⁴, wound infections⁵ and meningitis⁶. These hospital outbreaks are due to the ability of *A. baumannii* to survive in the hospital environment and to resistance to various antibiotics⁷. There are several factors contributing to the success of *A. baumannii* in survival in the hospital environment: (i) ability to form biofilm and adhere to biotic and abiotic surfaces such as environmental surfaces and medical devices⁸; (ii) ability to adhere to, colonize, and invade epithelial cells^{9,10}; (iii) ability to obtain foreign genetic materials to enhance its survival under host selection pressures and antibiotic treatment^{11,12} and (iv) its specific mechanisms of antibiotic resistance⁷. Among the mentioned factors, biofilm formation is the primary factor involved in bacterial resistance and survival¹³. Biofilm is a complex mixture of bacteria which is often enclosed with thick polysaccharide layer and is responsible for resistance to antibiotics^{14,15}. There are several virulence determinants which are associated with biofilm formation of *A. baumannii*. The present study was done to investigate the presence of various virulence factors and biofilm formation by clinical isolates of *A. baumannii*.

MATERIALS AND METHODS

Sample collection and identification of *A. baumannii*: In this study, a total of 120 clinical samples were collected from Namazi hospital in Shiraz, Iran. All *A. baumannii* isolates were isolated

from ICU (n=81), internal (n=30), and transplantation wards (n=9). The samples were transferred to brain-heart infusion broth medium and incubated aerobically for 24 h at 37°C. The turbid broth of the specimens was plated on blood agar (Merck, Germany) and MacConkey (Merck, Germany) for standard aerobic growth. Non-hemolytic and opaque colonies on blood agar and non-lactose fermentative colonies on MacConkey were subcultured on MacConkey agar and incubated for another 24h at 37°C to obtain pure colonies. *A. baumannii* species were identified by microscopic characteristics, Gram stain, and biochemical tests including IMVIC, catalase, urease, and oxidase. *A. baumannii* was confirmed using *bla*_{OXA-51-like} PCR assay¹⁶.

Detection of biofilm formation: Biofilm formation was assessed by microtiter plate method. For biofilm development, *A. baumannii* cultures were inoculated in trypticase soy broth (TSB) (Merck, Germany) with 20% glucose and adjusted to 0.5 McFarland standards. Each well of sterile 96-well polystyrene microtiter plates was filled with 200µl of bacterial suspension. The plates were aerobically incubated overnight at 37°C. Afterwards, the wells were tenderly washed three times with phosphate buffered saline (PBS) and stained for 5 min with 2% crystal violet. Excess crystal violet was rinsed off by water and

then air-dried. To resolubilize the dye bounded to the adherent cells, we the wells with 200µl of 33% glacial acetic acid. The optical density (OD) was measured at 570 nm, using ELISA reader. The OD results were grouped for biofilm formation as follows: (I) OD < 0.5 = non-biofilm producer; (II) 0.5 < OD < 1.5 = moderate biofilm producer; and (III) OD > 1.5 = strong biofilm producer.

Detection of virulence genes by PCR: Boiling method was used for the extraction of bacterial DNA from the isolates; the primer sequences are shown in Table 1. Polymerase chain reaction (PCR) was performed in a total volume of 25µl reaction

containing 5 µl of genomic DNA, 20 pmol of each of the primers, 200 µM of each dNTP, PCR buffer with 2.5 mM of MgCl₂, and 1.5 U of Taq DNA polymerase (Sinaclon). The PCR condition was as follows: 5 min of the first denaturation at 94°C followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose gels containing safe DNA stain.

Statistical analysis: SPSS 25.0 was used for statistical analysis. The Chi-square test and Fisher's exact 2-tailed test were performed for analysis of the data. Statistical significance was considered as <0.05.

RESULTS

Of the 120 clinical specimens, 100 *A. baumannii* were isolated (83.33%). All *A. baumannii* isolates were identified with

microscopic characteristics. Among the 100 *A. baumannii*, 52 of the isolates were from male patients and 48 from female patients. Presence of virulence factors was investigated by PCR assay. All isolates were analyzed for the presence of virulence genes; 10 (10%) had *iutA* gene and 5 (5%) had *papC* gene. Figure 1 illustrates the appearance of *iutA* and *papC*. However, *A. baumannii* isolates were negative for *cnf1*, *cnf2*, *fbn*, *kpsMT II* virulence factor genes.

Biofilm formation assay by microtiter plate method revealed 58% (n=58) of the isolates as biofilm producer, while 2(3%) of the isolates displayed strong biofilm formation, and 39% (n=39) indicated weak or no biofilm formation. In this study, the frequency of biofilm formation was significantly higher than the negative isolates ($P=0.028$). Table 2 presents the relationship between the presence of *iutA* and *papC* virulence factor genes and biofilm formation.

Fig 1; Patterns of agarose gel for detection of the virulence genes. Lane M: 100 bp ladder (Sinaclon), Lane C-: Negative control, Lane C+: Positive samples for *iutA* gene and *papC* gene, S: positive controls for *iutA* gene and *papC* gene

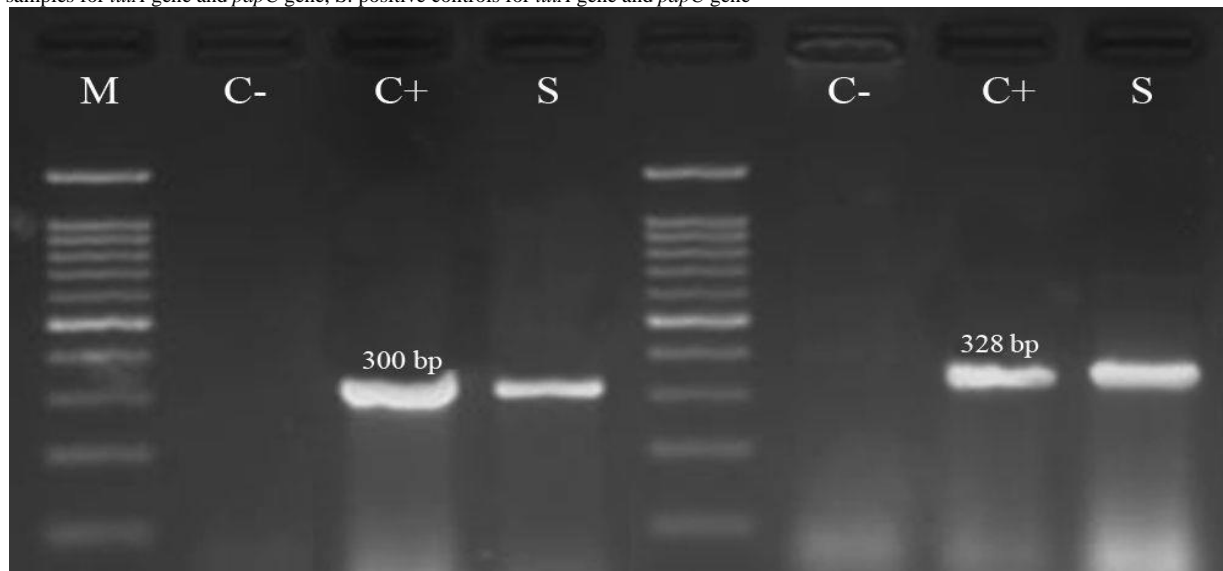


Table 1: Primers to virulence factor genes utilized in PCR

Gene	Virulence Factor	Primer	Sequence (5'-3')	Amplified DNA (bp)
<i>cnf1</i>	cytotoxic necrotizing factor	F	AAGATGGAGTTTCCTATGCAGGAG	498
		R	TTCAGAGTCCTGCCCTCATTATT	
<i>cnf2</i>	cytotoxic necrotizing factor	F	AATCTAATTAAGAGAAAC	543
		R	CATGCTTTGTATATCTA	
<i>fbn</i>	fibronectin receptor	F	GGTAATCAGTCATTCGAG	207
		R	TGGCACACTGTCCAAGTC	
<i>iutA</i>	aerobactin	F	GGCTGGACATCATGGGAAGTGG	300
		R	CGTCGGGAACGGGTAGAATCG	
<i>kpsMT II</i>	capsule	F	GCGCATTTGCTGATACTGTTG	272
		R	TCCAGACGATAAGCATGAGCA	
<i>papC</i>	P fimbriae	F	GACGGCTGTACTGCAGGGTGTGGCG	328
		R	TCCTTTCTGCAGGGATGCAATA	

Total	11(11%)	89(89%)	5(5%)	95(95%)
P value ^χ	0.957		0.401	

Table 2: The relationship between the presence of virulence factor genes and biofilm formation

Biofilm	<i>iutA</i>		<i>papC</i>	
	+ve	-ve	+ve	-ve
Negative	4(10.26%)	35(89.74%)	3(7.70%)	36(92.30%)
Moderate	7(12.06%)	51(87.94%)	2(3.34%)	56(96.55%)
Strong	0	3(100%)	0	3(100%)

DISCUSSION

HAIs (nosocomial infections) are known as the primary cause of death worldwide and the most important reason for nosocomial infections is Gram-negative bacteria¹⁷. *A. baumannii*, a Gram-negative bacteria, is an opportunistic pathogen which leads to

hospital outbreaks of infection due to multidrug resistance (MDR)¹⁸. Furthermore, the ability to adhere to biotic and abiotic surfaces and induce poor inflammatory responses in human cells are the other factors involved in the clinical success of *A. baumannii*¹⁹. Adhered cells could form biofilms, and biofilm increases the longevity of *A. baumannii* in hospital environments²⁰. The current study examined the presence of virulence factors in the isolated *A. baumannii* strains from three different wards of Namazi hospital in Shiraz, Iran. The ability of biofilm formation was also investigated.

We found that 83.33% of the samples were positive for *A. baumannii*. In agreement with our results, Pourhajibagher *et al.* showed that 83.3% of the samples from burned patients were positive for *A. baumannii*²¹. Momtaz *et al.* reported that 24.2% of their clinical samples in Iran were positive for *A. baumannii*²². Siau *et al.* reported 11% positive *A. baumannii* for the samples in the Hong Kong hospitals²³. In a study conducted in Iraq, the prevalence rate of *A. baumannii* was 18.75%²⁴. These differences in the prevalence rate of *A. baumannii* in different studies could be associated with various factors including the differences in the number of collected samples, a method of sampling, the ward of sampling, the type of samples, geographical areas, patients, age, and sex.

Our results showed that among the virulence factors, *iutA* (10%) and *papC* (5%) genes were detected in the isolated *A. baumannii* strains. These genes are primarily associated with iron uptake and adherence. Momtaz *et al.* found that the prevalence rates of *iutA* and *papC* virulence genes were 19.00% and 7.43%, respectively, which were slightly higher than our results. In contrast to our study, they reported the high prevalence of *cnfI* (35.53%), *cnf2* (25.61%), and *kpsMT II* (14.04%) virulence genes²². In a study by Darvishi, *cnfI* and *iutA* genes were positive in 50% and 25% of *A. baumannii* isolates from hospitalized patients in healthcare centers and hospitals of Iran, respectively²⁵. Similarly, AL-Kadmy *et al.* reported that the prevalence rates of *cnfI* and *iutA* genes in the *A. baumannii* isolates were 23.80% and 47.6%, respectively²⁴.

Among the one hundred isolated *A. baumannii*, 61 (61%) were able to form biofilm. Likewise, other studies showed that 62%²⁶ and 63%²⁷ of the isolated *A. baumannii* produced biofilm. Dehbalaei *et al.*'s study found that 72.91% of the isolated *A. baumannii* were able to form biofilm²⁸, which is higher than our results. Various results in biofilm production could be due to different methods. Adi-Ali *et al.* have used modified microtiter plate and test tube methods for assessment of biofilm formation. The obtained results of microtiter plate method were as follows: 25% negative, 41% weak, 10% moderate, and 18% strong. The results of the test tube were as follows: 18% negative, 42% weak, 18% moderate, and 22% strong²⁹.

CONCLUSION

In conclusion, we identified the high prevalence of *A. baumannii* isolated from Namazi hospital in Shiraz, Iran. Our findings revealed that *iutA* and *papC* virulence genes which are associated with iron uptake and fimbriae were positive among the isolates. We found that a large number of isolated *A. baumannii* can form biofilms which could guarantee their survival in hospital environment.

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Conflicts of interest: none

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