



Correlation of Virulence Factors and Cell Adhesion of Clinical Isolates of *Acinetobacter baumannii*

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Abstract

Background: Adhesion to host cells is an important and critical feature of *Acinetobacter baumannii* pathogenesis. *OmpA*, ABAYE1319-CsuA/B, and ABAYE2132 are highly prevalent and conserved fimbrial proteins; ABAYE1859 and ABAYE0304 fimbrial antigens are the factors described as novel vaccine candidates against multidrug-resistant (MDR) *A. baumannii*.

Methods: The current study aimed at investigating the significance of *A. baumannii* fimbrial and outer membrane proteins A (*OmpA*) in adherence to human epithelial cells. Antibiotic resistance was determined by the broth microdilution method. Phenotypic identification of isolates producing ESBL (extended-spectrum beta-lactamase) was conducted using double-disc synergy test (DDST). The virulence factors were detected by the polymerase chain reaction (PCR) in 100 non duplicate clinical isolates of *A. baumannii*; 18 clinical isolates were selected for adhesion assay based on antibiotic resistance and PCR results.

Results: The current study results showed that ABAYE2132, ABAYE0304, and ABAYE1319-CsuA/B had significant correlation with cell adhesion ($P \leq 0.05$). There was no significant correlation between cell adhesion and ABAYE1859 ($P \geq 0.05$). ABAYE2132 was the most effective factor in cell adhesion. It was also observed that cell adhesion rate reduced with the increase of fimbrial proteins.

Conclusions: The current study findings suggested that different products and structures of *A. baumannii*, most still unknown, play a pivotal role in pathogen-biotic reactions. Better exposure of some non-pili adhesins or other biofilm forming factors could be a reason for this phenomenon.

Keywords: Virulence Factors, *OmpA*, Cell Adhesion, Antibiotic Resistance, *Acinetobacter baumannii*

1. Background

Interaction of pathogen and host cell is critical in the pathogenesis of some microorganisms leading to their internalization (1, 2). In order to establish infections, bacteria should colonize the host. Attachment of pathogens to host cells is promoted by expression of various molecules or structures by bacterial cells. The adhesion depends on interactions of the receptors and host cell surface or soluble proteins playing a bridge role between bacteria and host cells (3). Microbial adherence to host cells as the initial step of the colonization process is an important virulence factor (4). *Acinetobacter baumannii*, recently emerged globally as an important nosocomial pathogen (5). Most of the strains are multidrug-resistant (MDR) with the ability to survive in the hospital environment and

spread among patients (6-8). *A. baumannii* strains cause pneumonia, bacteremia, meningitis, and wound and urinary tract infections (7, 9). In order to determine the outcome of infections more attention should be paid to the interaction between *A. baumannii* and the host epithelial cells (4, 10). The adherence potential of *A. baumannii* to human bronchial epithelial cells and its resistance to major antimicrobial agents explain its survival in the medical environment and the human host (11). A few molecular factors are required for *A. baumannii* virulence in humans. They include mainly Bap (biofilm-associated protein), *OmpA* (outer membrane protein A), phospholipase D, the Csu (chaperone-usher type pilus), the *Acinetobacter* trimeric autotransporter (Ata), and the acinetobactin-mediated iron acquisition system (12). Epidemiological

studies established the colonization and persistence of *Acinetobacter* species on human skin and mucosal membranes for days to weeks implying the ability of these microorganisms to adhere well to such surfaces (13, 14). *A. baumannii* adherence to host cells is mediated by fimbria or *OmpA* (1, 15). The exact mechanism of adherence of clinical *A. baumannii* isolates to various biotic or abiotic surfaces is not understood so far suggesting the possession of various independent molecular mechanisms for adherence to such surfaces by *A. baumannii* (16). It is reported that pili play an important role in twitching motility as well as adhesion and biofilm formation (17). *Acinetobacter* strains may construct more than one form of pili at time (18). Tomaras et al. (19), demonstrated pili formation ability of *A. baumannii* strain ATCC19606T and subsequently the bacterial adherence to abiotic surfaces via biofilm formation. This ability depends on the expression of a part of CsuA/BABCDE chaperone-usher pili assembly system known as *CsuE*. *OmpA* is the most prevalent surface protein in *A. baumannii* with 38 kDa molecular weight. This protein, associated with the permeability of small solutes, is responsible for adherence and subsequent invasion of *A. baumannii* in epithelial cells (15). In vitro adherence to epithelial cells is a useful study model to investigate the adhering capacity of microorganisms. There are only a few reports (1, 4, 9, 20) of in vitro adherence to epithelial cells by *Acinetobacter* species. Due to the limited number of effective antibiotics against MDRA. *baumannii* infections, the surface antigens could serve in the development of broadly protective vaccines (21).

2. Objectives

The current study aimed at investigating the correlation of virulence factors of clinical isolates of *Acinetobacter baumannii* and their ability to adhere bronchial epithelial cells and their adherence correlation with the virulence factors of clinical *A. baumannii* isolates through an in vitro bacterial adhesion assay.

3. Methods

3.1. Bacterial Strains

A. baumannii ATCC 19606 was used as standard strain and 100 clinical isolates were obtained from patients of Khatamolanbia hospital, Tehran, Iran in 2014. *A. baumannii* isolates were identified using standard biochemical and microbiological tests and confirmed by PCR amplification of 16S-23S ribosomal DNA intergenic spacer region (22).

The PCR using a pair of primers, PR-Ab-ITS (5'-TCTAAATTCACATACCAAACGC-3') and PF-Ab-ITS (5'-ACGGTAATTAGTGTGATCTGACG-3'), specifically amplified

an internal 166-bp fragment of the 16S-23S ribosomal DNA intergenic spacer region of *A. baumannii*. PCR reaction was performed in a 25 μ L total reaction mixture. Each reaction contained 0.4 μ M of each primer, 200 μ M of each dATP, dCTP, and dGTP, 190 μ M dTTP, 10 μ M DIG-11-dUTP (Roche Diagnostics), 0.5 U of Taq DNA polymerase, 2.5 μ L 10X buffer, 2 mM MgCl₂, and different concentrations of genomic DNA. PCR cyclic conditions were initiated with 95°C for three minutes followed by 35 cycles of 94°C for 45 seconds, 60°C for 40 seconds, 72°C for one minute, and a final extension at 72°C for five minutes. Finally, the PCR product was electrophoresed by 1% agarose gel.

3.2. Selection of *A. baumannii* Virulence Factors

OmpA, ABAYE1319- CsuA/b, fimbrial protein, and ABAYE2132-fimbrial protein (Group I), ABAYE1859-fimbrial protein (Group II), and ABAYE0304-fimbrial protein (Group III) were selected. Based on the prevalence and amino acid sequence conservation, the antigens were divided into three groups including highly prevalent and conserved antigens (Group I), highly prevalent and variable antigens (Group II), and low prevalent (Group III) (21).

3.3. Detection of Virulence Factors

Colony PCR, using the primers listed in Table 1, was performed with 20 μ L of master mix containing 5 U/ μ L Taq polymerase (0.2 μ L), 10X PCR buffer (2 μ L), 50 mM MgCl₂ (2 μ L), 10 pM reverse and forward primers (1 μ L each), and 1 μ L of dNTPs MIX (2 mM) in 9 μ L of distilled water.

3.4. Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined using broth microdilution method, based on the clinical and laboratory standards institute (CLSI) guidelines, in Mueller-Hinton broth (Merck, Germany) in 96-well plates with a serial dilution of antimicrobial agents ranging from 0.5 to 256 mg/L (23). The lowest dilution at which bacteria failed to grow was regarded as MIC value. *A. baumannii* isolates were tested against cefepime, ceftazidime, ceftriaxone, imipenem, meropenem, and gentamycin. *A. baumannii* ATCC19606 served as quality control (24).

3.5. Phenotypic Identification of Extended-Spectrum Beta-Lactamase Producing Isolates

Phenotypic identification of ESBL producing isolates was carried out with double-disc synergy test (DDST) screening method. The employed antibiogram disks included ceftazidime (30 μ g), cefotaxime (30 μ g),

Table 1. Primers Used in the Study

Primer	Primer Sequence (5' to 3')	Product Size (bp)	Target
Forward	ACGGTAATTAGTGTGATCTGACG	166	<i>Acinetobacter baumannii</i> genomic DNA containing 16S-23S IGS
Reverse	TCTAAATTCACATACCAAACGC		
Forward	TGGTCACTTGAAGCCTGCTG	208	OmpA
Reverse	TGCAGTAGCGTTAGGGTATTACG		
Forward	TATATTTCCACCGCATTACTTGATC	544	ABAYE2132-fimbrial protein
Reverse	AGCATAATAATTAAGATCGGCATTC		
Forward	ACTGGACAGGTTGACGTTAAATTG	432	ABAYE1319- CsuA/b; fimbrial protein
Reverse	TTTATAATCGCCTTGIGCTTTTG		
Forward	CGTGCGGTTAGTGAATCAGG	327	ABAYE0304-fimbrial protein
Reverse	ATATTTTCGGCAGCAGTACTTTC		
Forward	TTATATCGTGAAGCAGAGAATAGCAG	678	ABAYE1859-fimbrial protein
Reverse	TACAGTAGCCATTGCACGCAC		

ceftazidime-clavulanic acid (30/10 µg), and cefotaxime-clavulanic (30/10 µg). The Muller-Hinton agar (Merck, Germany) was used to place pairs of disks at 20 mm distance from each other. The P5 mm and growth inhibitory zone of ceftazidime-clavulanic acid and cefotaxime-clavulanic than ceftazidime and cefotaxime was regarded as an ESBLs producing isolate (25).

3.6. Adhesion Assay

The adhesion assay was performed as described by Gaddy et al. (26), using human lung epithelial cell line (A549). Each assay was repeated three times with fresh samples. Bacterial adherence was evaluated semi-quantitatively from zero to three (27) based on the following legend:

- Strong adherence (3): > 380 CFU/mL (10^4)
- Moderate adherence (2): 240 - 380 CFU/mL (10^4)
- Weak adherence (1): 120 - 240 CFU/mL (10^4)
- Very weak or no adherence (0): < 120 CFU/mL (10^4)

3.7. Statistical Analysis

SPSS version 21.0 was used for data analysis. Student *t* test was used to determine differences between means. Adherence data were compared employing the Mann-Whitney-Wilcoxon test. Correlations of adhesion and increased virulence factor were evaluated by general linear model analysis (multiple linear regression). P values < 0.05 were taken as statistically significant.

4. Results

4.1. Bacterial Strains and Selection of *A. baumannii* Virulence Factors

A. baumannii isolates were confirmed by PCR amplification of 16S-23S ribosomal DNA intergenic spacer region. As shown in Figure 1, PCR led to specific amplification of an internal 166-bp fragment of *A. baumannii* isolates.

4.2. Detection of Virulence Factors

The presence of *ompA* gene, as well as ABAYE1319, ABAYE2132, ABAYE1859, and ABAYE0304 (encoding fimbrial protein) was investigated by PCR (Figure 2). All the clinical *A. baumannii* isolates harbored *ompA* gene. Frequencies of the genes among 100 *A. baumannii* isolates were as follows: ABAYE2132 (88%), ABAYE1319 (84%), ABAYE1859 (51%), and ABAYE0304 (48%) (Table 2).

Table 2. Frequency of the Genes Among Clinical Isolates of *Acinetobacter baumannii*

Gene	Results	
	Positive (%)	Negative (%)
<i>Acinetobacter baumannii</i> genomic DNA containing 16S-23S IGS	100	0
ABAYE1319-CsuA/b; fimbrial protein	83	17
ABAYE0304-fimbrial protein	48	52
ABAYE2132-fimbrial protein	88	12
ABAYE1859-fimbrial adhesion	51	49
ABAYE0500-OmpA	100	0

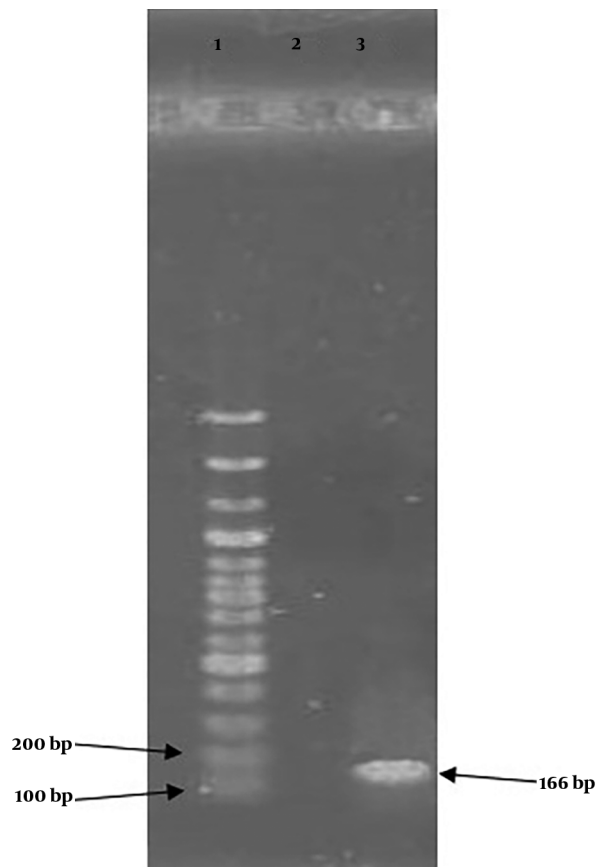


Figure 1. Identification of bacterial isolates by PCR. Lane1: A 100-bp DNA ladder; Lane2: Negative control; Lane3: *A. baumannii* genomic DNA containing 16S-23S IGS (166 bp).

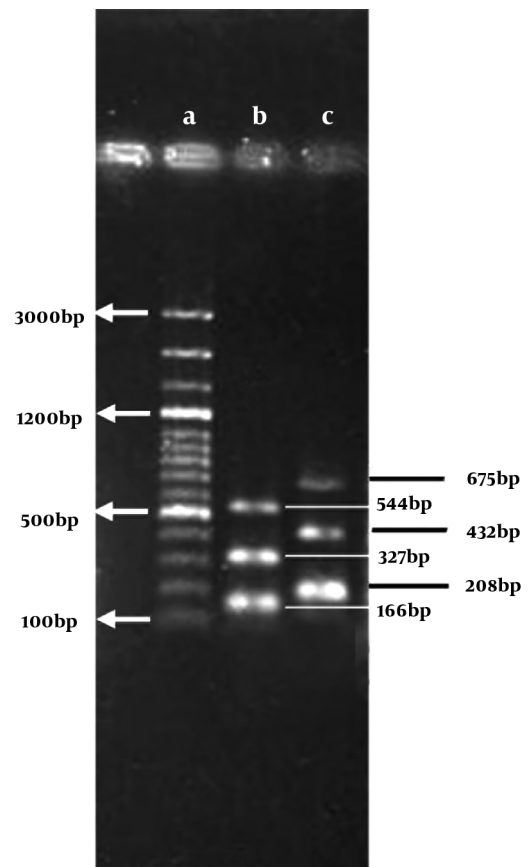


Figure 2. Detection of virulence factors in *Acinetobacter baumannii* isolate No.77 by multiplex PCR. Lane1: A 100-bp DNA ladder; Lane2: ABAYE2132-fimbrial protein gene (544 bp), ABAYE0304-fimbrial protein gene (327 bp), *A. baumannii* genomic DNA containing 16S-23S IGS(166 bp); Lane3: ABAYE1859-fimbrial protein gene (678 bp), ABAYE1319- CsuA/b; fimbrial protein gene (432 bp), *OmpA* (208 bp)

4.3. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility test revealed that 100% (n = 18) of the isolates were resistant to gentamicin, ceftriaxone, and ceftazidime. The resistance rates to cefepime and imipenem were 83.3% (n = 15) and 77.7% (n = 14), respectively. However, 22.2% (n = 4) of the isolates were susceptible to meropenem (MIC \leq 4 μ g/mL) as determined by the broth microdilution method. There was no significant correlation between virulence factors and the antibiotic resistance. Based on PCR results, 18 clinical *A. baumannii* isolates with one to four virulence factors were selected for antimicrobial susceptibility and adhesion tests (Table 3).

4.4. Phenotypic Identification of ESBL Producing Isolates

Phenotypic identification of ESBL producing isolates revealed that 11 (61%) isolates could produce ESBL enzymes. There was no significant correlation between virulence factors and ESBL producing isolates.

4.5. Adhesion Assay

All the 18 isolates attached to the A549 cells. The number of adhered bacteria varied from 35.6×10^4 to 447×10^4 CFU/mL (Figure 3). ABAYE2132, ABAYE0304, and ABAYE1319 had significant ($P < 0.05$) correlation with cell adhesion. Dispersion of virulence factors and their relevance to various strains of *A. baumannii* are illustrated in Figure 4. There was no significant correlation between cell adhesion and ABAYE1859 ($P \geq 0.05$). ABAYE2132 was the most effective factor in cell adhesion.

5. Discussion

Henrichsen and Blom (28) were the first to describe the pili presence on *Acinetobacter* strains in 1975 in *A. baylyi* BD4 producing bundle-forming thin pili measuring 2 - 3 nm in diameter. These pili were involved in adherence

Table 3. Evaluation of Adhesion Assay, Antibiotic Resistance, ESBL Production, and Virulence Factors in Clinical Isolates of *Acinetobacter baumannii*

Isolate No.	Adhered Bacteria ($\times 10^4$)	Frequency of Adhesion	Virulence-Associated Genes						MIC ($\mu\text{g/mL}$)					ESBL
			OmpA	CsuA/B	ABAYE2132	ABAYE1859	ABAYE0304	GEN	CRO	CAZ	FEP	MER	IMI	
83	447 \pm 14.29	11.2X	+	-	-	-	+	64	128	128	8	≤ 0.5	≤ 0.5	+
5	365 \pm 4.08	9.21X	+	-	-	+	+	64	128	128	64	16	64	-
96	320.6 \pm 16.36	8X	+	-	+	-	+	64	128	128	32	16	64	+
78	280 \pm 3.30	7X	+	-	+	+	+	64	64	128	16	16	32	+
89	261 \pm 4.08	6.59X	+	-	-	-	-	64	128	128	32	16	32	-
2	260 \pm 11.43	6.5X	+	+	+	-	-	64	64	256	32	4	16	+
62	213 \pm 2.87	5.3X	+	+	+	+	-	128	256	≥ 256	64	16	32	+
7	183.3 \pm 3.30	4.6X	+	+	+	+	+	64	64	128	32	32	32	-
53	143.3 \pm 6.65	3.6X	+	+	-	-	-	32	64	≥ 256	64	8	16	+
20	142 \pm 12.33	3.5X	+	+	+	-	-	64	64	≥ 256	64	16	32	+
19	136.6 \pm 4.99	3.4X	+	+	+	+	+	64	64	≥ 256	32	8	16	-
77	129 \pm 2.94	3.2X	+	+	+	+	+	64	64	≥ 256	128	32	32	+
1	81 \pm 6.16	2X	+	+	+	+	-	64	128	≥ 256	16	4	8	+
32	74.6 \pm 6.13	1.88X	+	+	+	-	-	128	64	≥ 256	32	8	32	-
87	71.6 \pm 1.25	1.8X	+	+	+	-	-	64	64	≥ 256	32	8	32	+
25	70 \pm 13.06	1.76X	+	+	+	+	-	64	128	≥ 256	128	≤ 0.5	≤ 0.5	+
61	51.3 \pm 2.62	1.29X	+	-	+	+	+	128	256	256	64	16	32	-
29	35.6 \pm 4.50	0.8X	+	+	+	+	+	32	128	128	64	8	8	+
<i>A.baumannii</i> ATCC19606 (positive control)	39.6 \pm 5.54	X	+	+	+	+	-	8	8	4	8	≤ 0.5	≤ 0.5	-

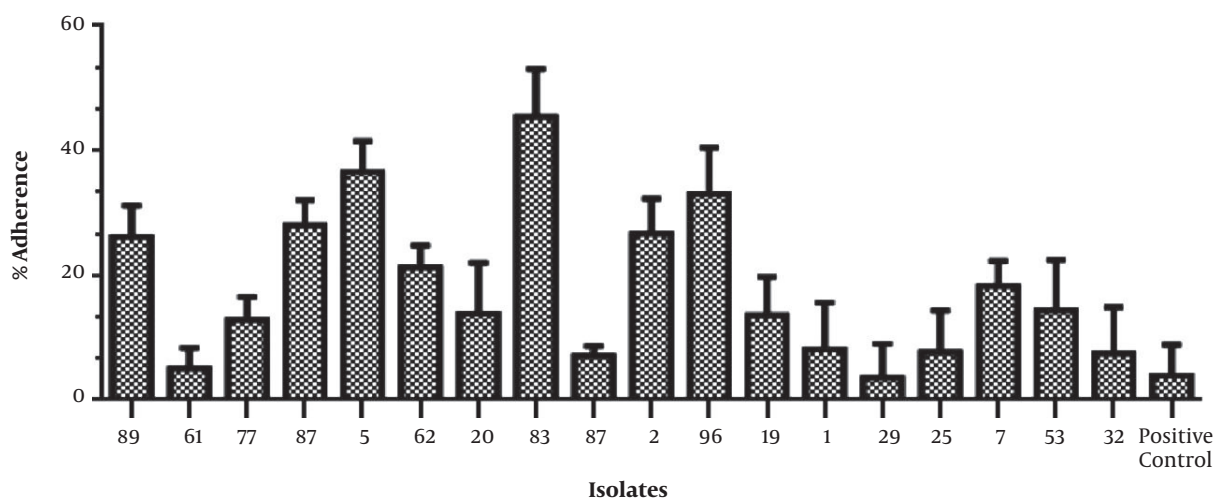


Figure 3. Adherence of clinical isolates of *Acinetobacter baumannii* to A549 cells

to biotic and abiotic surfaces. A 6-nm thick pilus was associated with twitching motility enabling colonization on adjacent surfaces (29). The current study employed surface antigens as virulence factors. These factors are already described as novel vaccine candidates against *MDR A. bau-*

mannii (21). Based on the prevalence and amino acid sequence conservation, the antigens could be classified into three groups with Group I antigens suggesting the most likely vaccine candidates (21). The current study results showed that ABAYE2132 (Group I) gene was more common

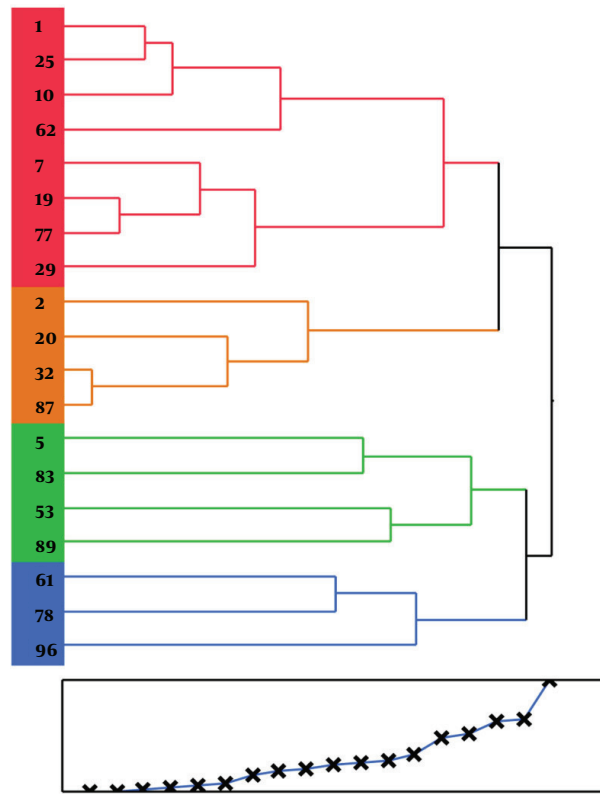


Figure 4. Dendrogram showing the relevance of virulence factors to *Acinetobacter baumannii* strains. Based on similar characteristics such as the presence of virulence factors and adherence to human bronchial epithelial cells, 18 clinical isolates were divided into four different groups

in the strains exhibiting stronger cell adhesion. Presence of this conserved and prevalent gene may be attributed to the virulence of *A. baumannii* strains. The obtained results were consistent with those of Moriel et al. (21). However, other fimbrial proteins in Groups II and III play a role in adherence and their use in a potential multi-component sub-unit vaccine cannot be ruled out since they are not highly prevalent or conserved (21). The adhesion of *A. baumannii* to the epithelium of human alveolar cells was also reported by Gaddy et al. (26), while describing the interaction of *A. baumannii* with other human respiratory cells. Adherence of *CsuE*-mutant strain rather than the parental strain to a number of epithelial cells could be attributed to the elevated expression of other surface elements such as different pilus types, as in *Escherichia coli* (29). The current study findings showed that *CsuA* was more effective in bacterial adherence to host cells. Dependence on the *CsuA*/BABCDE-mediated pili in interaction with biotic surfaces in contrast to abiotic surfaces was observed in the studied bacteria (26). Biofilm formation by *A. bauman-*

nii relies on the biosynthesis of pili; structures assembled through *CsuA*-BABCDE chaperone-usher secretion system (30). Overall observations in the current study indicated that various cell products and structures of *A. baumannii* were involved in interaction with biotic surfaces (26). In the current investigation, no significant correlation was observed between virulence factor genes and the antibiotic resistance. The current study results did not show any correlation between antibiotic resistance and cell adherence. In conclusion, the current study findings demonstrated that adherence to human epithelial cells is a general feature of *A. baumannii* and that ABAYE2132-fimbrial protein, a highly prevalent and conserved antigen, is involved in *A. baumannii*-biotic surface interaction. Further structural, physiological, and genetic studies are necessary to clarify the role of ABAYE2132-fimbrial protein in the pathogenesis of *A. baumannii*.

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Footnotes

Authors' Contribution: Iraj Rasooli: Laying out the core idea and designing the study, interpretation of data, and preparing the final copy of the manuscript; Maryam Afshar Payam: Performing the experiments; Parviz Owlia: Performing critical revision; Daryush Talei: Performing the statistical analyses; Shakiba Darvish Alipour Astaneh: Supervising the experiments; Shahram Nazarian: Drafting the manuscript

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