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ORIGINAL RESEARCH

Association Between Biofilm Formation, Structure, and the Expression Levels of Genes Related to biofilm formation and Biofilm-Specific Resistance of *Acinetobacter baumannii* Strains Isolated from Burn Infection in Ahvaz, Iran

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Background: The ability of biofilm formation is an effective way for *Acinetobacter baumannii* survival from stressed conditions. This present study was aimed to evaluate the association between biofilm formation, structure, the expression levels of genes related to biofilm formation and biofilm-specific resistance of *A. baumannii* strains isolated from burn infections in Ahvaz, Iran.

Methods: In this study, we assessed the antibiotic susceptibilities, ERIC-PCR typing, capacity of biofilm formation and biofilm structure of 64 *A. baumannii* isolates collected from burn infections. The distribution and the expression levels of genes involved in the biofilm formation including *bap, ompA, abaI, pgaA* and *csuE* were assessed by PCR and real-time PCR, respectively.

Results: We classified *A. baumannii* isolates in 14 clonal types of ERIC-PCR. Most *A. baumannii* isolates were resistant to all antibiotics tested except to tigecycline and colistin and had the biofilm formation capability but with different capacities. There was a significant inverse relationship between resistance to antibiotic agents and biofilm formation. The biofilm matrix of 50 strains consisted of polysaccharides together with DNA or proteins. The genes involved in the biofilm formation were detected in both biofilm-forming and non-biofilm forming; however, the expression levels of these genes were higher in biofilm producers compared with non-producers.

Conclusion: The biofilm cells exhibited dramatically decreased susceptibility to antibiotic agents; hence, they have great significance for public health. Therefore, the determination of antibiotic susceptibilities in biofilm and planktonic mode, molecular typing, and capacity of biofilm formation in clinical setting is essential.

Keywords: *Acinetobacter baumannii*, biofilm formation, gene expression, biofilm structure

Introduction

Burn infections are considered as one of the most important complications of thermal injuries. Burn infections may be a life-threatening cause if bacteria have invaded into the tissue layers beneath the dermis. In addition, the invasion of bacteria may lead to bacteremia, sepsis, and multiple-organ dysfunction syndrome.[1]

In addition to the nature of the burn infections, the quantities of microorganisms colonizing these infections are also important. Gram-negative bacteria due to their

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extensive antibiotic resistance have accounted as the main pathogens of the burn infections. *Acinetobacter baumannii* is recognized as one of the leading pathogens in burn units' worldwide.[2]

In recent years, the extensive emergence of multi- and pan drug-resistant *A. baumannii* strains has revealed this organism's ability to quickly adapt to environmental changes.[3]

The biofilm formation is one of the hallmark characteristics of opportunistic pathogens such as *A. baumannii*. Biofilm is a community of bacterial cells attached to biotic or abiotic surfaces where these cells have intimate contacts with each other. *A. baumannii* can produce a wide variety of virulence factors in the biofilm mode that contributes in the various steps of the attachment of biofilm cells to the biotic or abiotic surfaces.[4]

The biofilm-associated protein (Bap) is one of the key factors in the initial attachment and maturation of *A. baumannii* biofilm, so that can affect both the biofilm thickness and bio-volume.[5] Also, the major outer membrane protein (OmpA) has an important role in the attachment of bacterial cells on the abiotic surfaces and human alveolar epithelial cells in the biofilm communities.[6]

The production of pili is essential for the initial steps of biofilm formation. The pili of *A. baumannii* are encoded by the *csuA/BABCDE* operon and the inactivation of the *csuE* gene resulted in the abolition of both pili production and biofilm formation.[7] One of the important polysaccharides in the biofilm communities is poly-β-(1-6)-*N*-acetylglucosamine (PNAG) that its synthesis is controlled by the *pgaABCD* locus.[8] On the other hand, the production of the acyl-homoserine lactone molecules as the product of the autoinducer synthase *abaI* gene, is essential for quorum sensing and subsequently the later stages of the biofilm formation.[9] Overall, the presence and the expression levels of genes involved in the biofilm formation have determined the capacity of the biofilm formation.

One of the main characteristics of bacteria in the biofilm mode is greater tolerance to antibiotics rather than planktonic mode, so that bacteria in the biofilm mode can tolerate 100 to 1000 folds higher concentrations of antimicrobial agents than the planktonic mode.[10] Many studies in Iran and other countries reported the capacity of biofilm formation in *A. baumannii* strains, as well as the frequencies of genes related to it.[11–15] However, the associations between the expression levels of genes related to biofilm formation and the capacity of biofilm formation, as well as the structural properties of biofilm matrix are less studied. Hence, this study was aimed to evaluate the association between the biofilm formation, structure, the expression levels of genes related to biofilm formation and biofilm-specific resistance of *A. baumannii* strains isolated from burn infections in Ahvaz, Iran.

Methods

Study Design and Bacterial Strains

A total of 64 *A. baumannii* isolates were collected between August and December 2018 from clinical samples of burn infections of hospitalized patients in the intensive care unit (ICU) of Taleghani Hospital in Ahvaz, Iran. The study design was approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Iran (AJUMS. REC.1396.333). These isolates were transferred to Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences and cultured on MacConkey agar (Merck, Germany). All of these isolates were confirmed as *A. baumannii* by some biochemical tests, including sugar fermentation, motility, citrate utilization, urease, oxidative/fermentative glucose (O/F) test, catalase, oxidase and growth ability at 37°C and 42°C[16] and the amplification of the $bla_{\text{OXA51-like}}$ gene,[17] according to the primer set listed in Table 1. The *A. baumannii* strain ATCC19606 was used as the reference strain.

Antibiotic Susceptibility Testing

The minimum inhibitory concentrations (MICs) of a quinolone (levofloxacin), an aminoglycoside (amikacin), a carbapenem (meropenem), a glycylcycline (tigecycline), a fourth-generation cephalosporin (cefepime) and a lipopeptide (colistin) were performed using broth microdilution method and their results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSL, 2018).[18] All of these antibiotics were purchased from Sigma Aldrich, USA. Briefly, for levofloxacin, amikacin, meropenem, colistin and cefepime, the MICs of greater than or equal to 8 μg/mL, 64 μg/mL, 8 μg/mL, 4 μg/mL and 32 μg/mL were considered as the resistance breakpoints, respectively. In addition, for tigecycline, a MIC of greater than or equal to 8 μg/mL was proposed as the resistance breakpoint according to the criteria suggested by Jones et al.[19]

Molecular Typing by ERIC-PCR

The clonal relatedness of the *A. baumannii* strains was evaluated by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR).[20] This reaction was performed using primers ERIC-F (5′-ATGTAAGCTCCTGGGGATTCAC-3′)

and ERIC-R (5′-AAGTAAGTGACTGGGGTG AGCG-3′) in a final volume of 20 µL containing 2 U of Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer (forward and reverse), 10x PCR buffer, 2 µL template DNA (100 pg concentration) and nuclease-free water up to a final volume of 20µL. This amplification condition was as follows: one cycle initial denaturation at 94°C for 5 mins; 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1.5 mins, and a final extension cycle at 72°C for 15 mins. The comparison of ERIC-PCR banding patterns was performed using Gel Compare II software version 6.6 (Applied Math, Sint-Martens-Latem, Belgium). The relatedness of each ERIC-PCR pattern was determined using Dice coefficient/unweighted pair-group method with arithmetic mean [UPGMA] analysis, with 1% position tolerance. *A. baumannii* strains with a similarity of 85% were considered as a clonal type.

Biofilm Formation Determination and Quantification

The biofilm formation capability of *A. baumannii* isolates was assessed using the crystal violet (CV) staining method, as previously described.[21] Briefly, an overnight culture of each isolate was prepared in Muller Hinton broth and adjusted to a turbidity of 0.5 McFarland with normal saline (0.85% NaCl). A 10µL aliquot of each suspension was diluted 1:20 in 190 µL of Mueller Hinton broth containing 2.5% glucose in the 96-well polystyrene microtiter plates. After incubation at 37°C for an overnight, the plates were rinsed with PBS three times. The adherent cells were fixed with absolute methanol for 10 mins and stained with 200 µL of 0.1% CV for 20 mins at room temperature. Again, the plates were washed with PBS three times, and then air-dried. Subsequently, the unbound CV was removed by adding 200 µL of a mixture of ethanol:acetone (1:5) for 20 mins and the optical density (OD) at 570 nm was measured using an UV visible spectrophotometer (UV-1601, SHIMADZU). Also, *A. baumannii* strain ATCC19606 and Muller Hinton Broth were used as positive and negative controls for the biofilm formation, respectively. The results were interpreted according to the criteria suggested by Zhang et al.[22]

Determination of Biochemical Properties of the Biofilm Matrix

To characterize the biochemical properties of the biofilm matrix, first, the 24 hrs old biofilms of *A. baumannii* isolates were established in the sterile 96-well polystyrene microtiter plates and washed with PBS for three times. The biofilms were then treated for 1 hr at 37°C either with a solution of 10 mM sodium metaperiodate ($NaIO_4$) in 50 mM sodium acetate buffer for the disruption of the extracellular polysaccharides, with 100 µg/mL of proteinase K for the disruption of the extracellular proteins, or with 100 µg/mL of DNAseI in 150 mM of NaCl and 1 mM $CaCl_2$ for the disruption of the extracellular DNAs. Afterward treatments, the biofilms were washed with PBS, stained with 0.1% CV and the OD at 570 nm measured, as described above. Since the biofilm matrix for each isolate can be a combination structure from polysaccharide, protein and/or DNA, the composition of the biofilm matrix after removing biofilm with specific treatments of sodium metaperiodate ($NaIO_4$), proteinase K and DNAseI was described as + = ≤30%; ++ = 30–70% and +++ = ≥70%.[23]

Biofilm Antibiotic Susceptibility Testing

The minimum biofilm eradication concentration (MBEC) values of levofloxacin, amikacin, meropenem, tigecycline, colistin and cefepime in *A. baumannii* isolates were measured using the broth microdilution method. First, the isolates were cultivated in the sterile 96-well polystyrene microtiter plates for an overnight at 37°C to allow for the biofilm formation. The biofilms were then exposed to the concentrations of 2 to 4096 µg/mL of levofloxacin, 4 to 8192 µg/mL of amikacin, 2 to 4096 µg/mL of meropenem, 0.5 to 2048 µg/mL of tigecycline and 16 to 16,384 µg/mL of cefepime for an overnight at 37°C. Then, the wells were washed with sterile PBS three times, and incubated with Muller Hinton Broth (Merck, Germany) for an overnight at 37°C. The MBEC was proposed as any viable cell was not recovered from the biofilm material or i.e. OD_{570} was less than 0.1. All tests were repeated in triplicate.[21]

Molecular Identification of the Genes Encoding Biofilm Formation and Efflux Pumps

The genomic DNA of these isolates were extracted using the boiling method, as previously described.[17] The uniplex PCR assays were performed for the molecular identifications of the *bap, pgaA, abaI, ompA* and *csuE* genes and efflux pumps, including *adeB, adeJ* and *adeG* in a final volume of 25 µL.[15,24] The sequences and sizes of the primers used in this study are shown in Table 1.

The amplification mixture consisted of 1U Taq DNA polymerase (Cinaclone, Iran), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.35 µM of each primer, 10x PCR buffer, 5 µL of template DNA and distilled water up to a final volume

Table 1 Primers Used in This Study

Target	Primers	Length (bp)	Ref.
bla_{OXA-51}	F:TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353	17
pgaA	F: GCCGACGGTCGCGATAC R: ATGCACATCACCAAAACGGTACT	150	24
csuE	F: TCAGACCGGAGAAAAACTTAACG R: GCCGGAAGCCGTAT GTAGAA	150	24
bap	F: AATGCACCGGTACTTGATCC R: TATTGC CTGCAGGGTCAGTT	205	24
ompA	F: ATGAAAAAGACAGCTATCGCGATTGCA R: CACCAAAAGCACCAGCGCCCAGTTG	136	15
abaI	F: AATGCCTATTCCCTGCTCAC R: ATTGCTTCTTGCAGAATTGC	132	15
16 S rRNA	F: ACTCCTACGGGAGGCAGCAGT R: TATTACCG CGGCTGCTGGC	198	9

of 25 µL. The amplification process was performed in a Mastercycler Nexus Thermal Cycler Gradient (Eppendorf, Hamburg, Germany) with one cycle of initial denaturation at 94°C for 3 mins, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for the *bap, ompA* and *csuE* genes, 59°C for the *abaI* and *ompA* genes and 55°C for the *adeB, adeJ* and *adeG* genes for 30 s, extension at 72°C for 30 s, and a final extension cycle at 72°C for 10 mins. The PCR products were visualized on 2% agarose gel stained with safe stain.

Quantitative Real-Time PCR Assay

First, the RNA extraction and genomic DNA removal of all *A. baumannii* strains were performed using an RNeasy Mini kit (Qiagen, Tokyo, Japan). The quality and integrity of the total RNA were evaluated with the NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA) and agarose gel electrophoresis. The final concentration of each one of the RNA extracts was adjusted to 400 ng/µL. The RNA was reverse transcribed to cDNA using PrimeScript™ 1st strand cDNA Synthesis Kit (Qiagen Tokyo, Japan) according to the manufacturer's procedure and all of the cDNAs were stored at −20°C. Real-time PCR amplification reaction was prepared in a final volume 20 µL, with 400 ng cDNA, 10 µL RealQ Plus Master Mix Green (Amliqon, Denmark), 0.5 µL each of forward and reverse primers (10 nM each) and RNase- and DNase-free water up to the final volume 20 µL. The Primer sequences used for the genes related to the biofilm formation, including *bap, abaI ompA, csuE* and *pgaA* gene and efflux pumps, including *adeB, adeJ* and *adeG* are shown in Table 1.[24] The 16rRNA gene was used as an internal control for the normalization of gene expression levels. Real-time PCR was performed using a Step One Real-Time PCR System (Applied Biosystems, CA, USA) as follows: on cycle of initial denaturation at 95°C for 15 mins, 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

The relative expression levels of each gene in the clinical strains are shown as the fold changes compared with the transcript levels of those in *A. baumannii* strain ATCC 19606 and were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The descriptive statistical tests were used for the evaluation of OD values of the biofilm formation, the frequencies of genes encoding biofilm and susceptibility antibiotic pattern. Spearman's rank (r_S) correlation was used for the comparison of the biofilm formation capacity with susceptibility and resistance to antibiotic agents, and Pearson correlation coefficient (PCC) was used for the determination of correlation between MIC and MBEC values. The mRNA expression analysis were performed using Student's *t* test and one-way analysis of variance (ANOVA), followed by the Tukey's multiple comparison test. In all analyses, a two-sided significance level of <0.01 was considered statistically significant.

Results

Bacterial Strains, Antibiotic Susceptibility and Molecular Typing

A total of 64 non-duplicate isolates were confirmed as *A. baumannii* using the amplification of the bla_{OXA-51} gene. These samples were collected from the different clinical samples, including bronchial lavage 17 (26.56%), wound 15 (23.43%), tracheal secretion 11 (17.18%), urine 11 (17.18%), and blood 10 (15.62%). The antibiotic susceptibility of these isolates in planktonic mode was determined using the broth microdilution method and according to these results, 56.25% of the *A. baumannii* isolates were MDR. Moreover, the majority of the isolates were resistant to amikacin (79.7%; 51 isolates), followed by meropenem (75%; 48 isolates), levofloxacin (60.9%; 39 isolates), cefepime (53.1%; 24 strains) and tigecycline (25%; 16 isolates), while all isolates were sensitive to colistin. Also, according to the analysis of ERIC-PCR band patterns, these 64 *A. baumannii* isolates were clustered into 14 clonal types and 11 single type of ERIC-PCR. Figure 1 describes the dendrogram obtained from ERIC-PCR analysis of these isolates. The correlation between the MICs of each one of antibiotics and ERIC-PCR types is shown in Table 2. According to these results, all strains belonging to a same clone type had similar antibiotic resistance pattern, i.e. there was a significant association ($p< 0.05$) between the clone types and antibiotic resistance.

Biofilm Formation Capacity and Its Structure Characterizes

The biofilm-forming capacity of these 64 *A. baumannii* isolates was evaluated using CV staining method. The OD570 values for *A. baumannii* ACC19606 (as positive control) and Muller Hinton broth (as negative control) were 0.452 ± 0.052 and 0.085 ± 0.002, respectively. The OD570 values for the clinical strains ranged from 0.125± 0.056 to 1.745 ± 0.054. Overall, 55 isolates were biofilm producers among which, 15 (23.4%) isolates were strong producers, 13 (20.3%) were moderate producers and 27 (42.2%) were weak producers. Also, 13 (20.3%) isolates showed the biofilm formation more robust than *A. baumannii* ATCC19606. The correlation between biofilm-forming capacity and ERIC-PCR types is shown in Table 3. According to these results, all strains belonging to a same clone type had similar biofilm-forming capacity.

On the other hand, the structure characterizes of the biofilm matrix were recognized by several treatments. According to these treatments, the biofilm matrix in 5 (7.8%) *A. baumannii* isolates was exclusively composited from polysaccharides, in 15 (23.4%) isolates was composited from the combination of proteins, polysaccharides and DNA, in 25 (39.06%) isolates were composited from the combination of proteins and polysaccharides and in 10 (15.62%) isolates was composited from the combination of DNA and polysaccharides. The composition of the biofilm matrix for each strain is shown in Table 3. According to these results, all strains belonging to a same clone type had similar structure characterizes in biofilm matrix.

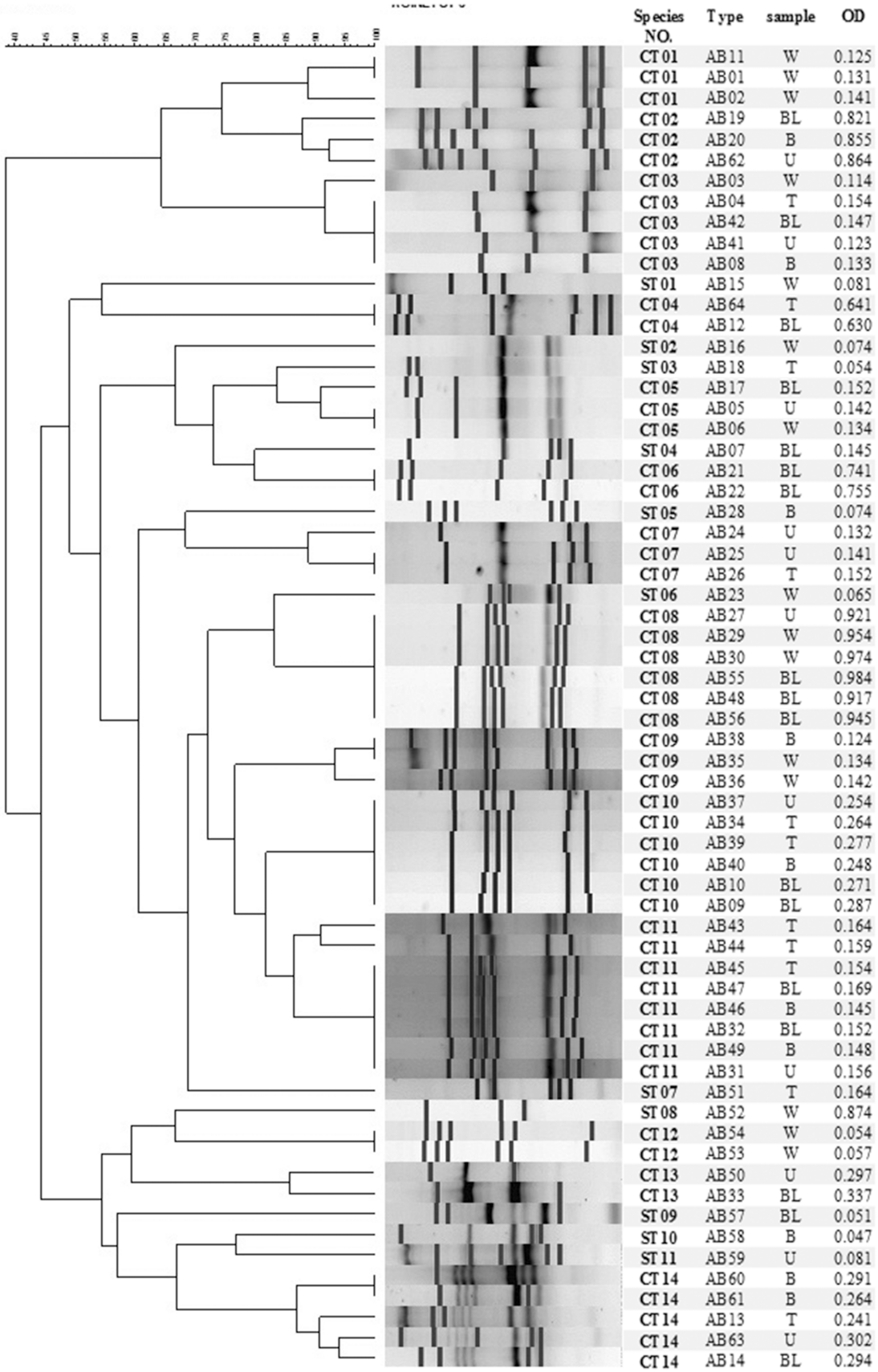
Relationships Between Biofilm Formation and Antibiotic Resistance

Our results revealed that all strong biofilm-formers were non-MDR strains, while all weak biofilm-formers were MDR strains. Also, nine strains were non biofilm-formers, which consisted of one non-MDR strain and eight MDR strains. Hence, it is suggested that non-MDR *A. baumannii* strains tended to form stronger biofilms than MDR strains, as was confirmed by statistical analyses ($r_s = -0.715$, $P < 0.001$), i.e. non-MDR strains had greater possibility to produce strong biofilm than MDR strains.

On the other hand, we evaluated the association between the biofilm-forming capacities and antibiotic resistance. The results revealed that apart from colistin, for each antibiotic, susceptible isolates could form more robust biofilms than moderate and resistant strains, indicating a significant negative correlation between the capacity of biofilm formation and resistance phenotypes to all antibiotics except to colistin ($r_s = -0.258$ to -0.708, $P < 0.001$; Table 4). For colistin, no significant correlation was observed ($r_s = -0.030$, $P = 0.813$; Table 4).

Relationship Between MBECs and MICs

The MIC values of meropenem, levofloxacin, cefepime, tigecycline, amikacin and colistin of these isolates ranged from 1 to 1024 μg/mL, 1 to 512 μg/mL, 0.5 to 512 μg/mL, 0.25 to 32 μg/mL, 8 to 512 μg/mL and 0.25 to 1 μg/mL, respectively. As expected, the MBECs of these antibiotics were higher than their respective MICs, followed by 32 to 4096 μg/mL for amikacin, 8 to 4096 μg/mL for levofloxacin and cefepime, 16 to 4096 μg/mL for meropenem, 8 to 512 μg/mL for tigecycline and 4 to 16 μg/mL for colistin.



Species NO.	Type	sample	OD
CT 01	AB11	W	0.125
CT 01	AB01	W	0.131
CT 01	AB02	W	0.141
CT 02	AB19	BL	0.821
CT 02	AB20	B	0.855
CT 02	AB62	U	0.864
CT 03	AB03	W	0.114
CT 03	AB04	T	0.154
CT 03	AB42	BL	0.147
CT 03	AB41	U	0.123
CT 03	AB08	B	0.133
ST 01	AB15	W	0.081
CT 04	AB64	T	0.641
CT 04	AB12	BL	0.630
ST 02	AB16	W	0.074
ST 03	AB18	T	0.054
CT 05	AB17	BL	0.152
CT 05	AB05	U	0.142
CT 05	AB06	W	0.134
ST 04	AB07	BL	0.145
CT 06	AB21	BL	0.741
CT 06	AB22	BL	0.755
ST 05	AB28	B	0.074
CT 07	AB24	U	0.132
CT 07	AB25	U	0.141
CT 07	AB26	T	0.152
ST 06	AB23	W	0.065
CT 08	AB27	U	0.921
CT 08	AB29	W	0.954
CT 08	AB30	W	0.974
CT 08	AB55	BL	0.984
CT 08	AB48	BL	0.917
CT 08	AB56	BL	0.945
CT 09	AB38	B	0.124
CT 09	AB35	W	0.134
CT 09	AB36	W	0.142
CT 10	AB37	U	0.254
CT 10	AB34	T	0.264
CT 10	AB39	T	0.277
CT 10	AB40	B	0.248
CT 10	AB10	BL	0.271
CT 10	AB09	BL	0.287
CT 11	AB43	T	0.164
CT 11	AB44	T	0.159
CT 11	AB45	T	0.154
CT 11	AB47	BL	0.169
CT 11	AB46	B	0.145
CT 11	AB32	BL	0.152
CT 11	AB49	B	0.148
CT 11	AB31	U	0.156
ST 07	AB51	T	0.164
ST 08	AB52	W	0.874
CT 12	AB54	W	0.054
CT 12	AB53	W	0.057
CT 13	AB50	U	0.297
CT 13	AB33	BL	0.337
ST 09	AB57	BL	0.051
ST 10	AB58	B	0.047
ST 11	AB59	U	0.081
CT 14	AB60	B	0.291
CT 14	AB61	B	0.264
CT 14	AB13	T	0.241
CT 14	AB63	U	0.302
CT 14	AB14	BL	0.294

Figure 1 Dendrogram of ERIC-PCR analysis of 64 *Acinetobacter baumannii* strains.

Table 2 MICs of Tested Antibiotics and ERIC-PCR Types for *Acinetobacter baumannii* Strains

Type	Strain No.	AK	CFP	LVF	TIG	MEM	COL	Type	Strain No.	AK	CFP	LVF	TIG	MEM	COL
		(μg/mL)								(μg/mL)					
CT01	AB11	64	128	8	16	64	1	CT08	AB56	16.0	4	4	0.5	2	0.5
CT01	AB01	64	128	8	16	64	0.5	CT09	AB38	128	512	128	8	8	0.25
CT01	AB02	128	128	8	16	128	0.25	CT09	AB35	128	256	128	4	8	0.25
CT02	AB19	128	64	1	2	1	1	CT09	AB36	64	256	128	8	8	1
CT02	AB20	128	128	1	2	1	1	CT10	AB37	128	8	8	2	256	1
CT02	AB62	128	64	2	2	1	0.5	CT10	AB34	128	16	8	1	256	0.25
CT03	AB03	256	512	16	8	256	1	CT10	AB39	128	16	8	4	128	0.25
CT03	AB04	256	512	16	8	512	0.5	CT10	AB40	64	8	4	1	128	1
CT03	AB42	256	256	16	4	512	0.5	CT10	AB10	64	16	4	4	128	1
CT03	AB41	256	512	32	4	512	0.25	CT10	AB09	128	16	4	2	256	0.5
CT03	AB08	256	512	16	8	256	0.25	CT11	AB43	128	128	64	16	512	0.5
ST01	AB15	16	16	32	4	512	1	CT11	AB44	128	128	64	4	512	0.5
CT04	AB64	16	4	2	0.5	2	0.5	CT11	AB45	256	128	64	8	1024	0.25
CT04	AB12	16	4	2	0.5	2	0.5	CT11	AB47	256	128	32	4	256	0.25
ST02	AB16	512	512	4	4	64	0.5	CT11	AB46	512	128	32	4	512	1
ST03	AB18	512	256	4	4	256	1	CT11	AB32	512	128	64	16	512	1
CT05	AB17	512	512	64	16	512	0.5	CT11	AB49	256	256	64	16	512	1
CT05	AB05	256	512	64	32	256	1	CT11	AB31	512	128	32	16	1024	0.25
CT05	AB06	512	256	64	16	256	0.5	ST07	AB51	64	512	64	0.5	64	0.25
ST04	AB07	64	512	128	4	512	0.25	ST08	AB52	16	2	512	1	64	0.5
CT06	AB21	8	4	1	4	4	0.25	CT12	AB54	512	1	4	4	64	0.5
CT06	AB22	8	4	1	4	4	0.5	CT12	AB53	512	0.5	4	4	128	0.25
ST05	AB28	256	16	4	1	512	0.5	CT13	AB50	64	1	128	1	2	0.25
CT07	AB24	512	128	64	4	256	0.25	CT13	AB33	64	2	128	0.5	2	0.25
CT07	AB25	512	128	64	4	128	0.25	ST09	AB57	16	1	64	0.5	1	0.5
CT07	AB26	512	256	128	4	128	1	ST10	AB58	256	16	4	0.5	256	1
ST06	AB23	256	16	4	4	128	0.25	ST11	AB59	512	8.	4	0.5	512	1
CT08	AB27	16	8	2	0.5	2	0.5	CT14	AB60	128	512	64	2	64	0.5
CT08	AB29	16	4	2	0.25	2	0.25	CT14	AB61	256	512	128	2	128	0.5
CT08	AB30	16	8	4	4	1	0.5	CT14	AB13	128	256	128	2	128	0.5
CT08	AB55	32	8	4	4	2	1	CT14	AB63	128	256	128	2	128	0.5
CT08	AB48	32	4	2	0.5	1	0.25	CT14	AB14	256	256	64	1	128	0.25

Abbreviations: CT, clonal type; ST, single type; AK, amikacin; LVF, levofloxacin; CEF, cefepime; TIG, tigecycline; COL, colistin; MEM, meropenem.

With an analysis of MBEC and MIC values of these antibiotics, we found an increase of 2- to 16-fold higher MBEC values rather than MIC values for amikacin, 4- to 32-fold for cefepime, colistin and meropenem, 8- to 32-fold for levofloxacin and 8- to 64-fold for tigecycline. By Pearson correlation coefficients (PCC) analysis of the MIC and MBEC values of each antibiotic, a significant positive relationship ($P < 0.001$) was demonstrated between the MIC and MBEC values for all of these antibiotics (PCC = 0.686 for tigecycline, PCC = 0.853 for cefepime, PCC = 0.885 for meropenem, PCC = 0.928 for levofloxacin and PCC = 0.799 for amikacin $P < 0.001$) except to colistin (PCC = 0.138; $P = 0.280$)

Distribution of the Genes Involved in Biofilm Formation and Efflux Pumps by PCR

The amplification of the genes encoding biofilm formation was performed by PCR. According to these results, all isolates (biofilm producers or no producers) harbored the *ompA* and *abaI* genes. Also, the majority of the isolates were positive for the amplification of *pgaA* gene (85.93%; 55 strains), followed by *csuE* gene (60.93%; 39 strains) and *bap* (48.43%; 31 strains). The presence of *csuE* gene was confirmed among all strong biofilm producers, 11 out of 13 intermediate producers, 3 out of 27 weak producers as well as 5 out of 9 no producers, while the *bap* and *pgaA* genes were detected only among biofilm producers. Moreover, the *pgaA*

Table 3 Biofilm-Forming Capacity (OD_{570}), Composition of the biofilm Matrix and ERIC-PCR Type of *Acinetobacter baumannii* Strains

Type	Strain	Capacity of Biofilm Formation	Removed Biofilm			Type	Strain	Capacity of Biofilm Formation	Removed Biofilm		
			NaIO4	PK	DNAseI				NaIO4	PK	DNAseI
CT01	AB11	Weak	++	+	+	CT08	AB56	Strong	+++	+	–
CT01	AB01	Weak	++	+	+	CT09	AB38	Weak	++	–	++
CT01	AB02	Weak	++	+	+	CT09	AB35	Weak	++	–	++
CT02	AB19	Strong	+++	+	–	CT09	AB36	Weak	++	–	++
CT02	AB20	Strong	+++	+	–	CT10	AB37	Intermediate	+++	+	–
CT02	AB62	Strong	+++	+	–	CT10	AB34	Intermediate	+++	+	–
CT03	AB03	Weak	+++	–	–	CT10	AB39	Intermediate	+++	+	–
CT03	AB04	Weak	+++	–	–	CT10	AB40	Intermediate	+++	+	–
CT03	AB42	Weak	+++	–	–	CT10	AB10	Intermediate	+++	+	–
CT03	AB41	Weak	+++	–	–	CT10	AB09	Intermediate	+++	+	–
CT03	AB08	Weak	+++	–	–	CT11	AB43	Weak	++	+	+
ST01	AB15	None	–	–	–	CT11	AB44	Weak	++	+	+
CT04	AB64	Strong	++	+	+	CT11	AB45	Weak	++	+	+
CT04	AB12	Strong	++	+	+	CT11	AB47	Weak	++	+	+
ST02	AB16	None	–	–	–	CT11	AB46	Weak	++	+	+
ST03	AB18	None	–	–	–	CT11	AB32	Weak	++	+	+
CT05	AB17	Weak	+++	+	–	CT11	AB49	Weak	++	+	+
CT05	AB05	Weak	+++	+	–	CT11	AB31	Weak	++	+	+
CT05	AB06	Weak	+++	+	–	ST07	AB51	Weak	+++	–	+
ST04	AB07	Weak	+++	–	+	ST08	AB52	Strong	++	+	+
CT06	AB21	Strong	++	++	–	CT12	AB54	None	–	–	–
CT06	AB22	Strong	++	++	–	CT12	AB53	None	–	–	–
ST05	AB28	None	–	–	–	CT13	AB50	Intermediate	++	++	–
CT07	AB24	Weak	++	++	–	CT13	AB33	Intermediate	++	++	–
CT07	AB25	Weak	++	++	–	ST09	AB57	Strong	+	++	+
CT07	AB26	Weak	++	++	–	ST10	AB58	None	–	–	–
ST06	AB23	None	–	–	–	ST11	AB59	None	–	–	–
CT08	AB27	Strong	+++	+	–	CT14	AB60	Intermediate	+++	–	+
CT08	AB29	Strong	+++	+	–	CT14	AB61	Intermediate	+++	–	+
CT08	AB30	Strong	+++	+	–	CT14	AB13	Intermediate	+++	–	+
CT08	AB55	Strong	+++	+	–	CT14	AB63	Intermediate	+++	–	+
CT08	AB48	Strong	+++	+	–	CT14	AB14	Intermediate	+++	–	+

Abbreviations: CT, clonal type; ST, single type; NaIO4, sodium metaperiodate; PK, proteinase K; + = ≤30%; ++ = 30–70% and +++ = ≥70%.

Table 4 Association Between the Biofilm-Forming Capacities and Antibiotic Resistance

Antibiotic Agents	Optical Density 570			r_s	P value
	Susceptible	Moderate	Resistance		
Amikacin	0.950(0.917–0.984)	0.687(0.051–0.974)	0.206(0.047–0.864)	−0.708	<0.001
Meropenem	0.748(0.741–0.755)	0.728 (0.051–0.984)	0.181(0.047–0.302)	−0.664	<0.001
Levofloxacin	0.809(0.630–0.954)	0.298(0.047–0.984)	0.192(0.051–0.874)	−0.258	<0.001
Tigecycline	0.458(0.047–0.954)	0.265(0.054–0.284)	0.141(0.114–0.164)	−0.479	<0.001
Cefepime	0.563(0.051–0.984)	0.219(0.054–0.864)	0.166 (0.047–0.287)	−0.406	0.001
Colistin	0.312(0.047–0.984)	–	–	−0.030	0.813

gene were recognized only among biofilm producers (55 strains), while the presence of *bap* gene was confirmed among all strong biofilm producers, 10 out of 13 intermediate producers and 6 out of 27 weak producers. Table 5 shows the association between the biofilm formation capacity, the clonal types and gene profiles. According to these results, all

Table 5 Association Between the Biofilm Formation Capacity, the Clonal Types and Gene Profiles

Capacity of Biofilm Formation	Gene Profile	ERIC-PCR Type	Frequency (%)
Strong	*Bap, CsuE, OmpA, pgaA, abaI*	CT02, CT04, CT06 CT08,ST08, ST09	15 (23.43)
Moderate	*CsuE,OmpA, pgaA, abaI* *Bap, OmpA, pgaA, abaI* *Bap, CsuE, OmpA, pgaA, abaI*	CT14,CT10 CT14 CT13, CT14, CT10	3 (4.6) 2 (3.1) 8 (12.5)
Weak	*OmpA, pgaA, abaI* *CsuE, OmpA, pgaA, abaI* *Bap,OmpA, pgaA, abaI*	CT01, CT03, CT11,ST04, ST07 CT05 CT07, CT09	18 (28.12) 3 (4.6) 6 (9.37)
None	*OmpA, abaI* *CsuE,OmpA, abaI*	ST01,ST02, ST05,ST10 CT12,ST11, ST06,ST03	4 (6.25) 5 (7.8)

biofilm strong producers had the gene profile of *bap, csuE, ompA, pgaA*,and *abaI*. Also, almost all of the strains belonging to a clonal type had similar gene profiles; however, the strains belonging to each one of the clonal types of CT10 and CT14 had several gene profiles, as described in Table 5.

On the other hand, the *adeB, adeJ* and *adeG* genes were detected among 92.18% (n=59), 98.43% (n=63) and 89.06% (n=57) of strains, respectively.

Expression Levels of the Genes Involved in Biofilm Formation and Efflux Pumps by Real-Time PCR

To determine the expression levels of the genes involved in biofilm formation and efflux pumps, we analyzed the rate of expression of each one of the genes involved in biofilm formation in 64 *A. baumannii* strains as compared to those in *A. baumannii* ATCC19606 strain by real-time PCR. Table 6 shows the expression means of the genes involved in biofilm formation with regard to the biofilm formation capacity. According to these results, the expression means of all of these genes were significantly higher among strong biofilm producers than moderate or weak producers ($p<0.01$).

As mentioned above, the *ompA* and *abaI* genes were detected among all isolates; however, the expression level means of these two genes were less among biofilm non-producers than biofilm producers (Table 6). Overall, there was a significant difference between the expression levels of all of the genes involved in biofilm formation and the biofilm formation capacity ($p<0.01$).

The expression levels of mRNA of the *adeB, adeJ* and *adeG* genes with regard to non-susceptible to antibiotics are described in Table 7. According to the results, the increased levels of *adeB* gene compared to that of *A. baumannii* ATCC 19606 were observed among 32 (50%) isolates ranged from 1.62- to 85.63-fold. The increased level of *adeB* was obvious among 23 (43.4%), 20 (44.4%), 25 (46.3%), 23 (60.5%) and 25 (50%) isolates of non-susceptible to amikacin, cefepime, levofloxacin, tigecycline and meropenem, respectively, while the level of *adeB* enhanced among 9 (81.8%), 12 (63.2%), 7 (70%), 9 (34.6%) and 7 (50%) isolates of susceptible to amikacin, cefepime, levofloxacin, tigecycline and meropenem, respectively.

The increased levels of *adeJ* gene compared to that of *A. baumannii* ATCC 19606 were observed among 16 (25%) isolates ranged from 1.5- to 1.96-fold. The increased level of *adeB* was obvious among 15 (28.3%), 14 (10.5%), 16 (26.6%), 7 (18.4%) and 15 (30%) isolates of non-susceptible to amikacin, cefepime, levofloxacin, tigecycline and meropenem, respectively, while the level of *adeB* enhanced among 1 (9.1%), 2 (31.1%), 0.9 (34.6%) and 1 (7.1%) isolates of

Table 6 Association Between the Expression Levels of Genes Involved in Biofilm Formation and the biofilm formation Capacity

Capacity of Biofilm Formation	*bap*	*ompA*	*pgaA*	*abaI*	*csuE*
Weak	0.65(0.54–0.75)	0.60(0.41–0.95)	0.71(0.54–0.91)	0.71 (0.62–0.85)	2.58(1.65–3.65)
Intermediate	1.91(1.43–2.41)	1.02(0.84–1.56)	1.18(0.82–1.95)	1.29(0.84–1.64)	0.72(0.54–0.95)
Strong	4.73 (2.65–6.31)	3.87(2.11–5.32)	3.87(2.65–4.62)	4.03(2.23–7.14)	4.41(2.92–7.45)
No biofilm	0	0.59(0.41–0.84)	0	0.50(0.45–0.63)	1.97(1.52–2.23)
P value	<0.01	<0.01	<0.01	<0.01	<0.01

Table 7 Expression Levels of mRNA of the *adeB, adeJ* and *adeG* Genes with Regard to Non-Susceptible to Antibiotics

Antibiotic	Group	MIC (μg/mL)	Gene Expression of		
			adeB	***adeJ***	***adeG***
Cefepime	Susceptible (n=19)	≤8	<1; n=3 1.31–9.47; n=10 10.22–85.63; n=6	<1; n=8 1.37–1.85; n=11	<1; n=9 1.39–4.65; n=3 10.1–69.35; n=5
	Non-susceptible (n=45)	16–128	<1; n=3 1.16–6.41; n=15 15.97–52.15; n=5	<1; n=11 1.23–1.96; n=12	<1; n=9 1.23–8.65; n=10 12.32–41.32; n=4
		≥256	<1; n=5 1.14–8.47; n=17	<1; n=10 1.23–1.89; n=12	<1; n=6 1.56–8.45; n=9 12.36–75.21; n=7
Amikacin	Susceptible (n=11)	≤16	<1; n=1 1.45–9.74; n=5 10.22–85.63; n=5	<1; n=3 1.37–1.85; n=8	<1; n=6 2.35–4.45; n=2 12.5–69.35; n=3
	Non-susceptible (n=53)	32–128	<1; n=5 1.16–6.41; n=16 18.64–52.15; n=3	<1; n=11 1.23–1.89; n=13	<1; n=8 1.32–8.65; n=13 12.59–41.32;n=3
		≥256	<1; n=5 1.14–8.47; n=22 15.97–30.41; n=2	<1; n=15 1.23–1.95; n=14	<1; n=12 1.23–3.76; n=7 10.1–75.21;n=10
Tigecycline	Susceptible (n=26)	≤2	<1; n=5 1.14–9.47; n=16 19.84–85.63; n=5	<1; n=7 1.23–1.89; n=19	<1; n=12 1.32–8.65; n=10 12.25–69.34; n=4
	Non-susceptible (n=38)	4–32	<1; n=6 1.14–8.74; n=26 10.22–37.14; n=6	<1; n=22 1.23–1.96; n=16	<1; n=14 1.23–8.45; n=12 10.1–75.21; n=12
Meropenem	Susceptible (n=14)	≤2	<1; n=3 1.14–9.47; n=6 34.14–85.63; n=5	<1; n=5 1.23–1.69; n=9	<1; n=8 1.39–8.69; n=3 12.25–69.35; n=3
	Non-susceptible (n=50)	4–64	<1; n=2 1.45–6.41; n=8 10.22–19.84; n=2	<1; n=7 1.5–1.86; n=5	<1; n=3 1.56–4.65; n=7 10.1–12.95; n=2
		128–1024	<1; n=6 1.14–8.74; n=28 15.97–37.14; n=4	<1; n=17 1.23–1.96; n=23	<1; n=15 1.23–8.45; n=12 10.65–75.21; n=11
Levofloxacin	Susceptible (n=10)	≤2	<1; n=1 1.16–9.47; n=4 10.22–85.63; n=5	<1; n=3 1.23–1.52; n=7	<1; n=3 2.36–8.65; n=4 12.25–69.35; n=3
	Non-susceptible (n=54)	4–32	<1; n=2 1.16–6.41; n=22 15.97–37.14; n=5	<1; n=18 1.23–1.96; n=11	<1; n=14 1.23–4.56; n=6 10.1–75.21; n=9
		64–512	<1; n=8 1.14–8.47; n=14 19.84; n=1	<1; n=8 1.25–1.89; n=17	<1; n=9 1.23–8.45; n=12 12.25–51.23; n=4

susceptible to amikacin, cefepime, levofloxacin, tigecycline and meropenem, respectively.

The increased levels of *adeG* gene compared to that of *A. baumannii* ATCC 19606 were observed among 32 (50%) isolates ranged from 1.56- to 75.21-fold. The increased level of *adeB* was obvious among 27 (50.9%), 25 (55.6%), 25 (46.3%), 20 (52.8%) and 27 (54%) isolates of non-susceptible to amikacin, cefepime, levofloxacin, tigecycline and meropenem, respectively, while the level of *adeB* enhanced among 5 (45.5%), 7 (36.8%), 7 (70%), 12 (46.2%) and 5 (35.7%) isolates of susceptible to amikacin, cefepime, levofloxacin, tigecycline and meropenem, respectively.

Statistical analysis revealed the significant association between the increased level of *adeB* gene and non-susceptibility to amikacin (p=0.043). However, no statistically significant association was found between overexpression of *adeB, adeJ* or *adeG* gene and non-susceptibility to other antibiotics.

The expression levels of mRNA of the *adeB, adeJ* and *adeG* genes with regard to the capacity of biofilm formation are described in Table 8. According to the results, the increased levels of *adeB* gene compared to that of *A. baumannii* ATCC 19606 were indicated among 4 (44.4%), 15 (55.8%), 3 (23.1%), and 10 (66.7%) isolates of non-biofilm producers, weak, intermediate, and strong producers, respectively. The increased levels of *adeG* gene compared to that of *A. baumannii* ATCC 19606 were indicated among 4 (44.4%), 15 (55.8%), 6 (46.2%), and 7 (46.7%) isolates of non-biofilm producers, weak, intermediated, and strong producers, respectively. The increased levels of *adeJ* gene compared to that of *A. baumannii* ATCC 19606 were indicated among 5 (55.6%), 5 (18.5%), 6 (46.2%), and 7 (46.7%) isolates of non-biofilm producers, weak, and intermediate producers, respectively, while the level of *adeB* enhanced among 22 (81.5%) weak producers and all strong producers.

Statistical analysis showed the significant association between the increased level of *adeJ* gene and the capacity of biofilm formation (p=0.004). However, no statistically significant association was found between overexpression of *adeB* or *adeG* gene and the capacity of biofilm formation.

Discussion

A. baumannii as one of the main causes of burn infections is responsible for an extensive range of serious infections.[25] The up-regulation of innate resistance mechanisms such as over-expression of efflux pumps and the acquisition of foreign genetic determinants such as plasmids is critical characteristics for the survival of *A. baumannii* during environmental pressures such as hospital environments.[26] The growing global emergence of *A. baumannii* strains resistant to all β-lactam agents, highlights the potential of this microorganism to adapt rapidly to selective environmental stresses. MDR *A. baumannii* is recognized to be among the most difficult antibiotic-resistant gram-negative bacilli to control and treat.[27]

The results of our study showed that 56.25% of *A. baumannii* isolates were MDR. The high prevalence of MDR *A. baumannii* isolates was also reported from some

Table 8 Expression Levels of mRNA of the *adeB, adeJ* and *adeG* Genes with Regard to Capacity of Biofilm Formation

Capacity of Biofilm Formation (Number)	Gene Expression Level					
	adeb	**(Number)**	***adeJ***	**(Number)**	***adeG***	**(Number)**
Non-biofilm (n=9)	1<; 1.32–6.34; 37.34;	n=1 n=7 n=1	1<; 1.65–1.96;	n=4 n=5	1<; 2.58–15.63;	n=5 n=4
Weak (n=27)	1<; 1.32–6.34; 37.34;	n=5 n=20 n=2	1<; 1.32–1.85;	n=16 n=11	1<; 1.32–8.45; 12.36–75.21;	n=8 n=10 n=9
Intermediate (n=13)	1<; 1.14–2.64; 18.64;	n=3 n=9 n=1	1<; 1.39–1.89;	n=3 n=10	1<; 1.32–3.78; 15.69;	n=5 n=7 n=1
Strong (n=15)	1<; 1.16–9.47; 10.22–85.63;	n=2 n=6 n=7	1<; 1.23–1.58;	n=6 n=9	1<; 2.36–8.45; 12.25–69.35;	n=8 n=4 n=3
P value	0.298		0.004		0.899	

studies in Iran and other countries.[28–33] The multidrug antibiotic resistance can decrease the efficacy of the common antibiotics used in the clinical setting especially in the infections caused by carbapenem-resistant strains. During the recent two decades, the carbapenems have recommended as the first-line antibiotics for the treatment of *A. baumannii* infections. However, unfortunately, increasing resistance to carbapenems has been reported worldwide among *A. baumannii* strains.[26] In our study, the antibiotic susceptibility test results showed that the majority of these isolates were resistant to meropenem (75%). In agreement with ours, the high rates of carbapenem-resistant *A. baumannii* isolates were reported from other studies.[28–33]

Also, our results showed that the antibiotic resistance rates to amikacin, cefepime and levofloxacin among MDR *A. baumannii* strains were more than 50%. In similar to our work, Mirnejad et al,[34] Huang et al[35] and Taherikalani et al[36] also, reported the high prevalence of the resistance to these antibiotic agents among *A. baumannii* isolates.

Tigecycline and colistin are the only treatment options for infections caused by extensively drug resistant (XDR) or MDR *A. baumannii*.[27] However, these two antibiotics have some undesirable side effects, such as nephrotoxicity and neurotoxicity. Nevertheless, carbapenems together with tigecycline or colistin are recommended as the best therapeutic approaches for the treatment of MDR *A. baumannii* infections. Moreover, these combination therapies are efficacious and have lower toxicity than monotherapy with tigecycline or colistin.[37]

The antibiotic susceptibility test results showed that the majority of *A. baumannii* isolates were susceptible to tigecycline and all isolates were sensitive to colistin which are in agreement with other reports obtained from previous studies in Iran and other countries.[38–41] Hence, these results suggest that tigecycline and colistin are still the most effective antibiotic agents against MDR *A. baumannii* strains.

The biofilm matrix can considerably protect bacteria from both the immune system cells and antibiotic agents.[42] In our study, most *A. baumannii* strains had the ability of biofilm production but with different capacities. As mentioned above, we found a significant inverse relationship between the capacity of biofilm formation and resistance to all antibiotic agents except to colistin ($p<0.001$), i.e. the biofilm density in sensitive strains was more than biofilm density in resistant strains. In consistent with our study, some researchers also[21,43,44] demonstrated that the sensitive strains tended to produce stronger biofilms than the resistant strains whereas some others[45,46] showed that MDR strains had more capability for the biofilm production than sensitive strains. It seems that the biofilm formation acts as a mechanism for bacteria to get a better survival when they are exposed to antibiotic agents, especially in the strains which their antibiotic resistance levels are not high enough.[21] The molecular mechanisms govern on this process are not clear yet; however, Gallant et al[47] showed that the expression of the β-lactamase gene bla_{TEM-1} in a clinical strain of *P. aeruginosa* inhibited the biofilm formation by distributing cell wall; indicating a genetic association between the biofilm formation and antibiotic resistance. Nevertheless, the exploration of the genetic links between biofilm formation and antibiotic resistance mechanisms than bla_{TEM-1} is required to fully elucidate the possible causes involved in this process.

The biofilm cells exhibit dramatically decreased susceptibility to antibiotic agents; hence, they have great significance for public health. In this present study, the MIC and MBEC values of antibiotic agents were determined using broth microdilution method. As expected, we found a significant increase in MBECs as compared to MICs for all of the antibiotic agents. These findings are also consistent with the findings of Li et al[21] indicating biofilm cells exhibit enhanced antibiotic resistance as compared to planktonic cells. The enhancement of biofilm-specific resistance can be explained by several factors, including the exopolysaccharide matrix of biofilm, different growth rates and nutrient gradients within the biofilm, mutational resistance, the up-regulation of efflux pumps, persister phenomenon and intrinsic characteristics of bacteria cells in biofilm mode.[10,48] Moreover, the persister cells as the dormant variants within bacterial biofilms have more tolerant to most antibiotics than planktonic bacteria that may cause a relapse of infection.[48]

Bacteria molecular typing is nowadays an integral part of the public health microbiology researches that is able to differentiate epidemiological relates from unrelated isolates belonging to a same bacterial species. Molecular typing is used to elucidate the source and route of transmission of microorganisms causing outbreaks of infectious diseases, the bacterial population structures and microbial genetic diversity in different environments. A variety of typing methods have been developed to classify and compare the genetic relatedness of bacterial isolates in epidemiological investigations[49] among which, ERIC-PCR is a plausible, easy and fast strategy that does not require the specialized equipment and reagents.[50] Our results showed that strains belonging to a clonal type had similar properties such as the composition of biofilm matrix, antibiotic resistance and gene

profile, indicating the importance of molecular typing methods such as ERIC-PCR for the identification of clonal types of *A.baumannii* in infectious diseases.

In *A. baumannii*, the genes encoding *ompA, bap, pgaA, csuE* and *abaI* are known to be the key factors in the biofilm formation. Our results revealed that the gene profile of *bap, csuE, ompA, pgaA, abaI* was presented in all biofilm strong producers and some moderate producers. Also, the gene pattern of *csuE, ompA, pgaA, abaI* was found in some biofilm weak producers and some moderate producers. These findings suggest that in addition to the presence of these genes, their expression levels also play the important role in the determination of the capacity of biofilm formation. On the other hand, some genes involved in the biofilm formation were detected in non-biofilm forming strains; however, the expression levels of these genes were higher in biofilm producers as compared with non-producers, which is in agreement with Wang et al[14] findings. Moreover, they indicated the higher expression levels of the *Bap1, AbaI* and *Csu A/B* genes in the biofilm-forming strains compared to the matched non-biofilm-forming strains.

The importance of efflux-mediated resistance in the development of resistance to several classes of antibiotics is indicated in several reports.[51–54] Our results indicated that the role of RND-type efflux pump of AdeABC in conferring to amikacin, as showed in the studies conducted by Magnet et al[55] and Marchand[56] et al who indicated the *adeB* mutants had more susceptibility (8 to 32-fold) to aminoglycoside agents than their parent strains; however, the expression level of AdeABC efflux pump was not measured in their studies. In this present study, no statistically significant association was found between overexpression of *adeB, adeJ* or *adeG* gene and non-susceptibility to other antibiotics. However, in contrast with our findings, some researchers revealed the high expression of the AdeABC efflux pump is closely associated with meropenem and tigecycline resistance,[57–59] suggesting still more researches are needful for elucidating the association between the AdeABC efflux pump and resistance to meropenem and tigecycline.

On the other hand, the efflux pumps especially the resistance-nodulation-cell division (RND) family confer low-level multidrug resistance and have several roles during the process of biofilm formation in *A. baumannii*. Moreover, these pumps have extruded actively the autoinducers associated with quorum sensing, and harmful molecules such as antibiotics and metabolic intermediates.[24] In this present study, there was a significant association between the overexpression of *adeJ* and the capacity of biofilm formation, so that all strong biofilm producers and 81.5% of intermediate producers showed the overexpression of the *adeJ*. However, similar to our study, no research has not showed the association between the expression of efflux pumps and the capacity of biofilm formation.

Conclusion

In this study, the frequencies of genes involved in biofilm formation were high. There was a significant inverse relationship between resistance to antibiotic agents and biofilm formation. Also, a significant increase in MBECs was showed as compared to their respective MICs. The genes involved in the biofilm formation were detected in both biofilm-forming and non-biofilm-forming strains; however, their expression levels were higher in biofilm producers as compared with non-producers.

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Disclosure

The authors report no conflicts of interest in this work.

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