Effects of sub-inhibitory concentrations of meropenem and tigecycline on the expression of genes regulating pili, efflux pumps and virulence factors involved in biofilm formation by Acinetobacter baumannii

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Background: Sub-minimal inhibitory concentrations of antibiotics have been indicated to affect the biofilm formation in pathogens of nosocomial infections. This study aimed to investigate the effects of meropenem and tigecycline at their sub-minimum inhibitory concentrations (MICs) on the biofilm formation capacity of Acinetobacter baumannii (A. baumannii), as well as the expression levels of genes involved in biofilm formation, quorum sensing, pili assembly and efflux pumps.

Materials and methods: In this study, four non-clonal strains (AB10, AB13, AB32 and AB55), which were different from the aspects of antibiotic susceptibility and biofilm formation from each other were selected for the evaluation of antimicrobial susceptibility, biofilm inducibility at sub-MICs of meropenem and tigecycline and the gene expression levels (the abaI, abaR, bap, pgaA, csuE, bfmS, bfmR, ompA, adeB, adeJ and adeG genes).

Result: A significant increase in the MICs of all antibiotics was demonstrated in the biofilm cells in each four strains. The biofilm formation was significantly decreased in all the representative strains exposed to tigecycline. However, the biofilm inducibility at sub-MICs of meropenem was dependent on strain genotype. In concordance with these results, Pearson correlation analysis indicated a positive significant correlation between the biofilm formation capacity and the mRNA levels of genes encoding efflux pumps except adeJ, the genes involved in biofilm formation, pili assembly and quorum sensing following exposure to meropenem and tigecycline at their sub-MICs.

Conclusion: These results revealed valuable data into the correlation between the gene transcription levels and biofilm formation, as well as quorum sensing and their regulation at sub-MICs of meropenem and tigecycline.

Keywords: Acinetobacter baumannii, sub-MIC, meropenem, tigecycline, biofilm formation, gene expression

Introduction

Acinetobacter baumannii is one of the opportunistic bacterial pathogens that primarily associated with a wide variety of hospital-acquired infections, particularly those who have hospitalized for a long time.¹ This bacterium has a high propensity to acquire a wide variety of antibiotic resistance determinants, as well as the capability of biofilm formation.
that these two characteristics play the important roles in treatment failure of this bacterium. Moreover, bacteria inside biofilms can tolerate the higher concentrations of antibiotics up to 1000 times more than their planktonic mode. The biofilm formation in A. baumannii is positively correlated with the transcription levels of several virulence factors, including two surface proteins of OmpA and Bap, the CsuABCDE operon that encodes type 1 pili, the pgaABCD locus that encodes proteins that synthesize cell-associated poly-ß-(1–6)-N-acetylglucosamine (PNAG) and the abaI gene that encodes acyl-homoserine lactones (AHL) as signal molecules.

Moreover, the CsuABCDE operon is one of the key factors in the biofilm formation of A. baumannii that is controlled by a two-component regulatory system of BfmS/BfmR. Previous findings indicated that BfmR is essential for the stabilization of the csu operon, especially the csuE gene, as well as the biofilm formation. On the other hand, the efflux pumps involved in multidrug resistance especially the resistance-nodulation-cell division (RND) family display several different roles during the transition of planktonic cell to biofilm in A. baumannii. Moreover, these pumps have extruded actively the autoinducers associated with quorum sensing, as well as harmful molecules such as antibiotics and metabolic intermediates, resulting in the regulation of the biofilm formation and quorum sensing processes directly and indirectly.

As described in previous studies, during the biofilm formation, a gradient of available substances such as oxygen, nutrient, pH, antibiotic is established; hence the cells within the inner layers of biofilm have a limited availability to the penetration of antibiotics, ie, these cells are exposed to sub-inhibitory concentrations of antibiotics. Moreover, several researchers showed that some antibiotics at sub-minimum inhibitory concentrations (MICs) can alter some bacterial functions such as the bacterial ultrastructure, the biofilm formation, the transcription of bacterial virulence factors and adhesions.

Carbapenems as a sustainable group of antibiotics with high activity and low toxicity are recommended for the treatment of infections associated with A. baumannii. However, in recent years, the emergence of the multidrug resistance A. baumannii (MDR-AB) isolates, which are resistant to carbapenems are increasing worldwide. So that the increasing resistance to carbapenems has limited their clinical use. Hence, the introduction of alternative antibiotic choices for the treatment of the MDR-AB infections is critical. Among antibiotic agents, polymyxins and tigecycline remain as the only active antibiotic choices against these infections. Moreover, a previous study by Sato et al indicated that colistin induced the biofilm formation in A. baumannii and increased the transcription levels of the genes associated with the biofilm. However, the effect of tigecycline at sub-MICs has already been not studied on the transcription levels of the genes associated with the A. baumannii biofilm. Hence, this current study was aimed to evaluate the effects of meropenem and tigecycline at their sub-MICs on the biofilm formation capacity of A. baumannii, as well as the expression levels of the genes involved in biofilm formation, efflux pumps and pili regulation.

Material and methods
Bacterial strains and antibiotic susceptibility
In this current study, four none-clonal strains (AB10, AB13, AB32 and AB55) based on ERIC-PCR patterns (data not shown) were selected for more analysis. Moreover, these four strains had differed from each other in aspects of the antibiotic susceptibility and the biofilm formation capability, as mentioned in Table 2. Identification of these isolates was performed using standard biochemical tests and confirmed by the amplification of bldOXA-51-like gene. The study design was approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS.REC.1396.333), Iran.

Biofilm formation determination and quantification
The biofilm formation capability of A. baumannii isolates was evaluated using the crystal violet staining method in the 96-well polystyrene microtiter plates, as previously described. Also, A. baumannii 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.

Antibiotic susceptibility testing
The minimum inhibitory concentrations of levofloxacin, amikacin, meropenem, tigecycline and cefepime were determined using broth microdilution method and their results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). Briefly, for levofloxacin, amikacin, meropenem, and cefepime, the MICs of greater than or equal to 8, 64,
8 and 32 µg/mL are considered as the resistant breakpoints, respectively. In addition, for tigecycline a MIC of greater than or equal to 8 µg/mL is proposed as the resistant breakpoint according to the criteria suggested by Jones et al.22

**Biofilm antibiotic susceptibility testing**

The minimum biofilm eradication concentration (MBEC) values of levofloxacin, amikacin, meropenem, tigecycline and cefepime in *A. baumannii* isolates were measured using the broth microdilution method.19 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 2–4,096 µg/mL of levofloxacin, 4–8,192 µg/mL of amikacin, 2–8,192 µg/mL of meropenem, 0.5–2048 µg/mL of tigecycline and 16–16,384 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, Darmstadt, Germany) for an overnight at 37°C. The MBEC was proposed as any viable cell was not recovered from the biofilm material or, ie, OD of 570nm (OD_{570}) was <0.1. All tests were repeated in triplicate.

**Biofilm formation in the presence of sub-MICs of tigecycline and meropenem**

First, each strain was inoculated in the 96-well polystyrene microtiter plates at approximately 10⁶ CFU/ml in cation-adjusted Mueller–Hinton broth with the different sub-inhibitory concentrations (1/8, 1/4 and 1/2×the MIC) of either tigecycline or meropenem. Then, the plates were incubated at 37°C for an overnight and the quantification of biofilms was performed as mentioned in the previous section. The antibiotic-free medium in well was used as negative control. Also, *A. baumannii* ATCC19606 was used as the positive control strain for the biofilm formation in the presence of sub-MICs of tigecycline and meropenem. The results were described as the OD_{570} ratio of the sub-MICs, ie, the 1/8×MIC, 1/4×MIC or 1/2×MIC of tigecycline or meropenem to the OD_{570} of control sample (0 MIC).23

**Quantitative real-time PCR assay**

First, these four representative *A. baumannii* strains were exposed to sub-inhibitory concentrations of either tigecycline or meropenem as described in before section. Then, RNA extraction was performed using an RNeasy plus Mini kit (Qiagen, Tokyo, Japan). The quality and integrity of the total RNA were evaluated with the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoresed on 1% agarose gel. The final concentration of the RNA extracts of these four strains was adjusted to 400 ng/µL. The RNA was reverse transcribed to cDNA using PrimeScript™ 1st strand cDNA Synthesis Kit (Qiagen) according to the manufacturer’s procedure (Transgen Bio-Technology Company, Beijing, China). The cDNA was kept 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) and 0.5 µL each of forward and reverse primers (10 nM each) and RNase- and DNase-free water up to in the final volume 20 µL. The primer sequences used for the genes involved in biofilm formation (*bap, ompA, csuE* and *pgaA*), quorum sensing (*abaI* and *abaR*), pili regulation (*bfmS* and *bfmR*) and efflux pumps (*adeB, adeG* and *adeJ*) are shown in Table 1.4,5,23,24 The 16s rRNA gene was used as a internal control for the normalization of the mRNA expression. 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 fold changes of mRNAs were calculated using the 2^(_ΔΔCt_) method. The relative expression of each gene after the exposure of the bacteria at sub-MICs of meropenem and tigecycline was normalized to the control sample (0 MIC), which was assigned a value of 1 arbitrary unit.

**Statistical analysis**

The mRNA expression analysis was performed using Student’s *t* test and one-way ANOVA, followed by the Tukey multiple comparison test. Pearson correlation analysis was used to analyze the gene expression levels and biofilm formation as well as quorum sensing. In all analyses, a two-sided significance level of <0.05 was considered statistically significant.

**Results**

**Antibiotic susceptibility of strains in planktonic and biofilm mode**

The values of MIC and MBEC of these representative four strains to antibiotic agents mentioned above is shown in Table 2. According to these results, the MIC
The values of meropenem, levofloxacin, cefepime, tigecycline and amikacin of these strains ranged from 2 to 512 µg/mL, 4 to 64 µg/mL, 8 to 256 µg/mL, 2 to 16 µg/mL and 32 to 512 µg/mL, respectively. As expected, the MBECs of these antibiotics were higher than their respective MICs, followed by 512 – 8192 µg/mL for amikacin, 128 – 4,096 µg/mL for cefepime, 128 – 1,024 µg/mL for levofloxacin, 256 – 4,096 µg/mL for meropenem and 64 – 512 µg/mL for tigecycline. With analysis of MBEC and MIC values of these antibiotics, we indicated an increase of 16-fold higher MBEC values rather than MIC values for amikacin, 8- to 128-fold for meropenem, 8- to 16-fold for cefepime, 8- to 64-fold for levofloxacin and 32- to 64-fold for tigecycline.

Table 1 Primers used in this study

| Gene  | Primer 5' to 3'                          | Ref. |
|-------|------------------------------------------|------|
| abaI  | F-CCGCCTTCCTCTAGACGTCGTA              | 4    |
|       | R- AAAACCCGACAGCTGTAATTA               |      |
| pgoA  | F- GCAGCAGGTCGCCGCACA                  | 4    |
|       | R-ATGGACATACGACGCCGTTACT               |      |
| csuE  | F- TCAGACGCGGAGAAAACTTAACG             | 4    |
|       | R- GCCGGAACGCGTGTGTAAGA                |      |
| bab   | F- AATTGCACCGTTGCA                     | 4    |
|       | R- TATTGCCGTTGATGTCGTT                 |      |
| 16S rRNA | F-ACTTCCTACGGAGGAGCGACGT             | 4    |
|       | R-TATTACGGCGCTGCTGTC                   |      |
| bfmS  | F- ACACGGGTAATCGAAG                    | 5    |
|       | R-TGATACCTATTCGCCGCTTTA                |      |
| bfmR  | F- GCTTTACGGGTTTTCGTG                 | 5    |
|       | R- GTAGTTGAACGGTGTTGC                  |      |
| adeB  | F- GCTTTTTCGACTGACACCCCAA             | 23   |
|       | R- GTGAGATTATTTTGCTGTC                 |      |
| adeJ  | F- GGTTCATTATACGCTGTC                 | 23   |
|       | R- GTGAGATTATTTTGCTGTC                 |      |
| adeG  | F- TTCATATGGCAAGGAGAGA                | 23   |
|       | R- GTGATGCGACCTGCTGTC                  |      |
| aboR  | F- ACCTTTTGGTTCGTCAGTC                | 24   |
|       | R- CGTGCTTCTCCCTCAAAAT                |      |

Abbreviations: MIC, minimum inhibitory concentration; MBEC, minimum biofilm eradication concentration.

Table 2 Antibiotic susceptibility of strains in planktonic and biofilm mode

| Strain | Meropenem MIC | Meropenem MBEC | Amikacin MIC | Amikacin MBEC | Tigecycline MIC | Tigecycline MBEC | Levofoxacin MIC | Levofoxacin MBEC | Cefepime MIC | Cefepime MBEC |
|--------|---------------|---------------|--------------|---------------|----------------|-----------------|----------------|----------------|--------------|--------------|
| AB10   | 128           | 2048          | 64           | 1024          | 4              | 128             | 4              | 128            | 16          | 256          |
| AB13   | 64            | 512           | 128          | 2048          | 2              | 64              | 4              | 256            | 1           | 256          |
| AB32   | 512           | 4096          | 512          | 8192          | 16             | 512             | 4              | 128            | 1           | 1024         |
| AB55   | 2             | 256           | 32           | 512           | 4              | 256             | 4              | 128            | 8           | 128          |

Abbreviations: MIC, minimum inhibitory concentration; MBEC, minimum biofilm eradication concentration.

Effects of sub-MICs of tigecycline and meropenem on the biofilm formation

The greatest ability of the biofilm formation in the absence of antibiotics was belonged to strain AB55 (OD_{570} = 0.984), followed by strain AB10 (OD_{570} = 0.271), strain AB13 (OD_{570} = 0.241) and strain AB32 (OD_{570} = 0.152). Figure 1 demonstrates the biofilm formation capacity of the representative strains in the presence of levofloxacin and meropenem at 1/8, 1/4, and 1/2× the MICs rate to the biofilm formation in the absence of these antibiotics.

For strain AB55, following exposure to tigecycline, the biofilm formation was decreased significantly at concentrations of 1 and 2 µg/mL by 0.65- and 0.68-fold changes, whereas meropenem induced significantly the biofilm formation at concentrations of 0.25 µg/mL (15.64-fold), 0.5 (14.35-fold) and 1 µg/mL (12.33-fold).

For strain AB10, the significant decrease of the biofilm formation was observed in the presence of tigecycline at both the concentrations of 0.5 and 1 µg/mL, resulting in 0.65- and 0.58-fold changes, respectively. Also, following exposure to meropenem, the biofilm formation induced significantly at concentration of 16 µg/mL (a 2.23-fold change), whereas reduced significantly at concentration of 64 µg/mL (a 0.78-fold change).

For strain AB13, the significant decrease of the biofilm formation was observed in the presence of tigecycline at concentrations of 0.5 and 1 µg/mL (by 0.52- and 0.69-fold changes) and meropenem at both concentrations of 16 and 32 µg/mL (by 0.62- to 0.78-fold changes).

For strain AB32, the significant decrease of the biofilm formation was observed in the presence of tigecycline at concentrations of 2 and 4 µg/mL, resulting in 0.73- and 0.57-fold changes, respectively. However, meropenem induced significantly the biofilm formation in a concentration-dependent manner, resulting in the changes of 15.64-, 14.35-, 12.34-fold at the concentrations of 64, 128 and 256 µg/mL respectively.
Expression levels of genes regulating pili, efflux pumps and virulence factors involved in the presence of sub-MICs of tigecycline

Figure 2 shows the effect of tigecycline at sub-MICs on the expression levels of the efflux pumps, pili regulation and biofilm involved genes in *A. baumannii* strains.

For strain AB55, the gene expression levels of the *bap* (0.68-fold), the *abaI* (0.68-fold), the *abaR* (0.58-fold) were significantly decreased at the concentration of 0.25 µg/mL, as well as the *pgaA* (0.55- and 0.60-fold) and the *adeB* (0.57- and 0.69-fold) at concentrations of 0.25 and 0.5 µg/mL, respectively. However, the relative
**Figure 2** Effect of tigecycline at sub-MICs on the expression levels of the efflux pumps, pili regulation and biofilm involved genes in *A. baumannii* strains. Error bars represent the standard deviations; *significant difference at a P-value of 0.05.*

**Abbreviations:** *A. baumannii*, *Acinetobacter baumannii*; MIC, minimum inhibitory concentration.
expression levels of the *ompA*, *bfmS*, *bfmR*, *csuE* and *adeJ* genes were not significantly changed at any concentration (*P*>0.05).

For strain AB10, the significant decreases in the relative expression levels were observed for the *bap* (0.63-fold), the *csuE* (0.46-fold), the *adeB* (0.78-fold), the *adeG* (0.66-fold), the *bfmS* (0.61-fold) and the *bfmR* (0.52-fold) at concentration of 0.5 µg/mL, the *ompA* (0.43-fold) at the concentration of 0.5 µg/mL, as well as the *abaI* (0.63- and 0.73-fold), the *abaR* (0.55- and 0.50-fold) and the *pgaA* (0.71- and 0.56-fold) at both concentrations of 0.5 and 1 µg/mL, respectively. However, the relative expression level of the *adeJ* gene was not significantly changed at any concentration (*P*>0.05).

For strain AB13, the significant decreases in the relative expression levels were observed for the *csuE* gene (0.76-fold), the *pgaA* (0.67-fold), the *adeG* (0.58-fold), the *bfmS* (0.75-fold) and the *bfmR* (0.55-fold) at the concentration of 1 µg/mL, the *bap* (0.57- and 0.77-fold) and *adeB* (0.58- and 0.75-fold) at both the concentrations of 0.5 and 1 µg/mL, respectively; as well as the *abaI* (0.41- to 0.74-fold) and the *abaR* (0.53- to 0.75-fold) in a concentration dependent manner (0.25–1 µg/mL). However, the relative expression levels of the *ompA* and *adeJ* genes were not significantly changed at any concentration (*P*>0.05).

For strain AB32, the significant decreases in the relative expression levels were indicated for the *bap* (0.63-fold), the *abaI* (0.70-fold) and the *adeG* (0.86-fold) at concentration of 2 µg/mL, as well as the *csuE* (0.75- and 0.57-), the *pgaA* (0.58- and 0.47-fold), the *adeB* (0.72-and 0.64-fold), the *abaR* (0.70- and 0.55-fold), the *bfmS* (0.87- and 0.55-fold) and the *bfmR* (0.81- and 0.63-fold) at both concentrations of 2 and 4 µg/mL, respectively. However, the relative expression levels of the *ompA* and *adeJ* genes were not significantly changed at any concentration (*P*>0.05).

Expression levels of genes regulating pili, efflux pumps and virulence factors involved in the presence of sub-MICs of meropenem

Figure 3 shows the effect of meropenem at sub-MICs on the expression levels of the efflux pumps, pili regulation and biofilm involved genes in *A. baumannii* strains.

For strain AB55, the significant increases in the relative gene expression levels were observed for all of genes except the *adeJ* gene in a concentration-dependent manner (0.25–1 µg/mL).

For strain AB10, the significant increases in the relative gene expression levels were observed for the *bap* (2.41-fold), the *csuE* (2.19-), the *pgaA* (2.11-fold), the *ompA* (2.3-fold), the *abaI* (3.18-fold), the *abaR* (4.11-fold) and the *bfmS* (2.23-fold), the *bfmR* (2.56-fold), the *adeB* (4.43-fold) and the *adeG* (3.21-fold) at the concentration of 16 µg/mL. However, the gene expression level of the *adeJ* was not significantly changed at any concentration (*P*>0.05).

For strain AB13, the significant decreases in the relative gene expression levels were observed for the *abaR* (0.75-fold) and the *adeB* (0.72-fold) at the concentration of 32 µg/mL, the *bap* (0.48- and 0.73-fold), the *abaI* (0.62- and 0.83-fold), the *bfmS* (0.62- and 0.79-fold), the *bfmR* (0.60- and 0.80-fold) and the *csuE* (0.55- and 0.76-fold) and the *adeG* (0.58- and 0.69-fold) at both concentrations of 16 and 32 µg/mL, respectively. However, the relative expression levels of the *ompA*, *pgaA* and *adeJ* were not significantly changed at any concentration (*P*>0.05).

For strain AB32, the significant increases in the relative gene expression levels were observed for the *bap*, *pgaA*, *csuE*, *abaI*, *abaR*, *bfmS*, *bfmR*, *adeB* and *adeG* genes at each three concentrations in a concentration-dependent manner (64–256 µg/mL). Moreover, a significant increase in the relative expression level of the *ompA* (1.81- and 1.63-fold) was observed at both the concentrations of 64 and 128 µg/mL, respectively; whereas the gene expression of the *adeJ* was not significantly changed at any concentration (*P*>0.05).

Correlation between biofilm formation and gene expression

To understand the correlation between the biofilm formation and the relative gene expression levels, we calculated the Pearson correlation coefficients between the capability of biofilm formation and the relative expression levels of the target genes (*bap*, *ompA*, *csuE*, *pgaA*, *abaI*, *abaR*, *bfmS*, *bfmR*, *adeB*, *adeG* and *adeJ*) for four strains of AB10, AB13, AB32, and AB55 exposed to sub-MICs of tigecycline (Table 3) and meropenem (Table 4).

In the presence of tigecycline, a significant positive correlation was indicated between the biofilm formation capacity and the gene expression levels of the *bap*, *pgaA*, *csuE*, *abaI*, *abaR*, *bfmS*, *bfmR*, *adeB* and *adeG* in two strains of AB13 and AB32. Also, there was a significant correlation between the biofilm formation capacity and the expression levels of the *bap*, *pgaA*, *abaR*, *adeG* and *adeB* gene in strain of AB55. In addition, for strain of AB10, the biofilm formation capacity was
Figure 3 Effect of meropenem at sub-MICs on the expression levels of the efflux pumps, pili regulation and biofilm involved genes in A. baumannii strains. Error bars represent the standard deviations; *significant difference at a P-value of 0.05.

Abbreviations: A. baumannii, Acinetobacter baumannii; MIC, minimum inhibitory concentration.
highly correlated with the expression levels of all target genes except the adeJ gene.

In the presence of meropenem, a significant positive correlation was indicated between the biofilm formation capacity and the expression levels of all genes except the adeJ in three strains of AB55, AB32 and AB10. Also, the capability of biofilm formation was highly correlated with the expression levels of the bap, csuE, abal, abar, bfmS, bfmR, adeB and adeG genes in strain of AB13.

Correlation between quorum sensing and gene expression

To understand the correlation between the quorum sensing and the relative gene expression levels, we calculated the Pearson correlation coefficients between the capability of quorum sensing and the relative expression levels of the target genes (bap, ompA, pgaA, csuE, abar, bfmS, bfmR, adeB, adeG and adeJ) for four strains of AB10, AB13, AB32, and AB55 exposed to sub-MICs of tigecycline (Table 5) and meropenem (Table 6).

In the presence of tigecycline, a significant positive correlation was indicated between the quorum sensing (abal) and the gene expression levels of the bap, pgaA, csuE, abar, bfmS, bfmR, adeB and adeG in two strains of AB13 and AB32. Also, there was a significant correlation between the quorum sensing and the expression levels of the bap, pgaA, abar and adeB gene in strain of AB55. In addition, for strain of AB10, the quorum sensing or the expression level of abal gene was highly correlated with the expression levels of all target genes except the adeJ gene.

In the presence of meropenem, a significant positive correlation was indicated between the quorum sensing and the expression levels of all genes except the adeJ in two strains of AB55 and AB10. Also, for strain of AB13, the capability of biofilm formation was highly correlated with the expression levels of the bap, csuE, abar, bfmS, bfmR, adeB and adeG genes in strain of AB13.

**Table 3** Association between biofilm formation and the gene expression profiles of *A. baumannii* strains at sub-MICs of tigecycline

| Strain | bap    | ompA   | csuE   | pgaA   | abal   | abar   | bfmS   | bfmR   | adeB   | adeG   |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| AB10   | 0.965* | 0.886* | 0.963* | 0.990* | 0.974* | 0.990* | 0.957* | 0.911* | 0.962* | 0.382  |
| AB13   | 0.997* | 0.506  | 0.906* | 0.971* | 0.994* | 0.988* | 0.936* | 0.950* | 0.994* | 0.448  |
| AB32   | 0.957* | 0.522  | 0.962* | 0.997* | 0.937* | 0.983* | 0.974* | 0.965* | 0.963* | 0.523  |
| AB55   | 0.897* | 0.484  | 0.224  | 0.992* | 0.897* | 0.932* | 0.270  | 0.231  | 0.997* | 0.302  |

**Table 4** Association between biofilm formation and the gene expression profiles of *A. baumannii* strains at sub-MICs of meropenem

| Strain | bap    | ompA   | csuE   | pgaA   | abal   | abar   | bfmS   | bfmR   | adeB   | adeG   |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| AB10   | 0.975* | 0.989* | 0.770* | 0.976* | 0.911* | 0.887* | 0.977* | 0.953* | 0.764* | 0.033  |
| AB13   | 0.994* | 0.116  | 0.990* | 0.049  | 0.974* | 0.998* | 0.964* | 0.978* | 0.992* | 0.516  |
| AB32   | 0.989* | 0.834* | 0.977* | 0.983* | 0.978* | 0.980* | 0.964* | 0.987* | 0.924* | 0.083  |
| AB55   | 0.986* | 0.902* | 0.789* | 0.836* | 0.891* | 0.932* | 0.892* | 0.872* | 0.924* | 0.259  |

**Table 5** Association between Quorum sensing with the gene expression profiles of *A. baumannii* strains at sub-MICs of tigecycline

| Strain | bap    | ompA   | csuE   | pgaA   | abar   | bfmS   | bfmR   | adeB   | adeG   |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| AB10   | 0.884* | 0.955* | 0.943* | 0.937* | 0.981* | 0.888* | 0.814* | 0.877* | 0.440  |
| AB13   | 0.991* | 0.497  | 0.943* | 0.981* | 0.984* | 0.957* | 0.959* | 0.985* | 0.416  |
| AB32   | 0.946* | 0.247  | 0.983* | 0.951* | 0.975* | 0.978* | 0.997* | 0.953* | 0.248  |
| AB55   | 0.998* | -0.444 | 0.212  | 0.929* | 0.982* | -0.445 | 0.259  | 0.891* | 0.339  |

**Table 6** Association between Quorum sensing with the gene expression profiles of *A. baumannii* strains at sub-MICs of meropenem

| Strain | bap    | ompA   | csuE   | pgaA   | abar   | bfmS   | bfmR   | adeB   | adeG   |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| AB10   | 0.884* | 0.955* | 0.943* | 0.937* | 0.981* | 0.888* | 0.814* | 0.877* | 0.440  |
| AB13   | 0.991* | 0.497  | 0.943* | 0.981* | 0.984* | 0.957* | 0.959* | 0.985* | 0.416  |
| AB32   | 0.946* | 0.247  | 0.983* | 0.951* | 0.975* | 0.978* | 0.997* | 0.953* | 0.248  |
| AB55   | 0.998* | -0.444 | 0.212  | 0.929* | 0.982* | -0.445 | 0.259  | 0.891* | 0.339  |
genes. In addition, for strain of AB32, the quorum sensing was highly correlated with the expression levels of all target genes.

Discussion

Acinetobacter baumannii has emerged as one of the opportunistic pathogens causing nosocomial infections. The emergence of MDR strains as one of the main consequences of antibiotics excessive use in the treatment of human infections, compromises a major challenge to health systems worldwide. While most previous studies have investigated the different mechanisms of antibiotic resistance in A. baumannii, but there are few studies that evaluated the effects of antibiotics at sub-MICs on the biofilm formation and pathogenicity of A. baumannii. Hence, this study was aimed to investigate the effects of two antibiotics of meropenem and tigecycline on the biofilm formation capacity, as well as the expression levels of the genes involved in biofilm formation, efflux pumps and pili regulation in A. baumannii.

In this study, we indicated a significant increase of MBEC values compared to MIC values. This enhancement of MBEC values can be due to several factors such as the exopolysaccharide matrix of biofilm, overexpression of efflux pumps, persister biofilm cells and intrinsic characteristics of biofilm cells. Furthermore, the persister cells are metabolically dormant and are usually present in the stationary phase, as well as biofilm. These cells are extremely tolerant to antibiotics without undergoing any genetic change and may cause a relapse of infection.

Carabapenems (meropenem and imipenem), as a class of β-lactam antibiotics, are increasingly being used as first-line therapy of serious hospital-acquired infections. In the current study, we evaluated the effect of meropenem at sub-MICs on the biofilm formation capability in the four representative A. baumannii isolates. According to our results, meropenem induced significantly the capability of biofilm formation in two representative strains of AB55 and AB32, whereas decreased the biofilm formation in strain of AB13. Also, in AB10 strain, the biofilm formation was induced at the concentration of 1/8× the MIC while was decreased at the concentration of 1/2× the MIC. In agreement with our results, He et al demonstrated the different effects of meropenem at its sub-MICs on biofilm formation capability of non-clonal A. baumannii strains, indicating that meropenem has affected the biofilm formation dependent on strain type and highlight the importance of molecular typing methods prior to the choice of antibiotic therapy.

In this study, following exposure to sub-MICs of tigecycline, the ability of biofilm formation was decreased significantly in two strains of AB13 and AB55 at both concentrations of 1/4 and 1/2×the MIC, as well as two strains of AB32 and AB10 at both concentrations of 1/8 and 1/2×the MIC. Inconsistent with our results, Maestre et al and Chen et al reported that tigecycline at its sub-inhibitory concentrations interfered with forming biofilm by E. faecalis and A. baumannii strains, respectively. However, in contrast to our results, Szczuka et al and Weiser et al indicated that tigecycline induced forming biofilm by S. epidermidis through overexpression of extracellular matrix binding protein (Embp) and other biofilm-associated genes, suggesting that the effects of sub-MICs of tigecycline are almost dependent on bacterial species. In our study, tigecycline at sub-MICs decreased significantly the biofilm formation in these four representative strains whereas meropenem decreased significantly the biofilm formation only in two representative strains, suggesting that tigecycline rather than meropenem can interfere with the induction of biofilm formation in A. baumannii strains. Hence, exposure to the sub-MIC doses of tigecycline in patients is more effective than meropenem in killing A. baumannii strains without undergoing any effect on induction of biofilm formation.

In A. baumannii, Abal/AbaR quorum sensing system is responsible for the synthesis and recognition of the AHLs. Following binding of the AHLs to AbaR, this conjugate binds

| Strain     | bap  | ompA | csuE  | pgaA | abaR | bfmS | bfmR | adeB | adeJ | adeG |
|------------|------|------|-------|------|------|------|------|------|------|------|
| AB 10      | 0.911* | 0.956* | 0.974* | 0.856* | 0.998* | 0.947* | 0.993* | 0.905* | -0.142 | 0.971* |
| AB 13      | 0.979* | -0.002 | 0.991* | -0.050 | 0.970* | 0.997* | 0.996* | 0.988* | 0.476 | 0.992* |
| AB 32      | 0.974* | 0.831* | 0.998* | 0.935* | 0.998* | 0.916* | 0.998* | 0.930* | 0.754* | 0.993* |
| AB 55      | 0.953* | 0.969* | 0.993* | 0.940* | 0.993* | 0.995* | 0.995* | 0.996* | -0.098 | 0.891* |
| ATCC19606  |      |      |       |      |      |      |      |      |      |      |

Note: Significant P-value at the level of 0.05 (2-tailed).

Abbreviations: A. baumannii, Acinetobacter baumannii; MIC, minimum inhibitory concentration.
to specific promoter DNA elements and regulate transcription of
target genes such as genes involved in biofilm formation.37
Our results demonstrated a significant positive correlation
between the expression levels of the abal and abaR genes
and biofilm formation at the sub-MICs of meropenem and
tigecycline, suggesting a strong association between quorum
sensing and forming biofilm by A. baumannii. Concordant to
our results, previous studies also,16,23 confirmed
a considerable correlation between the overexpression of the
abal gene and the biofilm formation when A. baumannii was
exposed to levofloxacain, meropenem and colistin.

In addition, we studied the correlation between three
RND efflux pumps of AdeABC, AdeFGH and AdeIJK
with the biofilm formation, as well as quorum sensing.
Our results indicated a significant positive correlation
between the overexpression of the adeB and adeG genes
and increased biofilm formation at the sub-MICs of meropenem and
tigecycline in these four representative strains,
that was in agreement with results obtained from the studies
of Sato et al16 and He et al23 when A. baumannii was
exposed to sub-MICs of antibiotics.

On the other hand, the up-regulation of the adeB and
adeG genes was positively correlated with the transcription
level of abal gene, indicating a strong link between the
RND efflux pumps (AdeABC and AdeFGH) and quorum
sensing.

It seems that the overexpression of AdeABC and
AdeFGH facilitate the transport of AHLs, resulting in the
increase of the biofilm formation in A. baumannii. Hence,
the inactivation of these two efflux pump by efflux pump
inhibitors (EPIs) might be an alternative treatment
approach to inhibit A. baumannii biofilm formation.23,38
In this study, the expression level of the adeJ gene was
much low, so that any significant correlation was not found
between the expression level of the adeJ gene and biofilm
formation in these strains that is in agreement with the
results of He et al23 on A. baumannii biofilms. Moreover,
several studies confirmed that the overexpression of the
adeJ gene is lethal for the host; hence its expression is
strictly regulated by A. baumannii biofilms.39–41

Our results showed that the transcription level of the
csuE gene together with its regulatory genes, bfms and
bfmr, were positively correlated with the biofilm formation
in all representative strains in the presence of either
meropenem or tigecycline. Moreover, Tomaras et al42 and
Pakharukova et al43 proved that the presence of type I pili
on the surface of A. baumannii is critical in the early step of
the biofilm formation on abiotic surfaces. So that the
disruption of the csuC and csuE genes through direct muta-
genesis resulted in non-piliated cells and abolishing the
ability of the biofilm formation. Also, we indicated that
the mRNA levels of the csuE gene together with the bfms
and bfmr were positively correlated with quorum sensing
and implicitly the expression of the abal gene. Also, the
upregulation of the csuE gene was concordant to the expression
levels of the Bfms and Bfmr genes, as demonstrated by
Luo et al5 Moreover, the researchers had proved that the
increased expression of the Bfms and Bfmr genes enhanced
the expression level of the csu locus and subsequently
forming pili for twitching motility in A. baumannii. Also,
in support of our findings, they indicated the increased
expression of all genes belonging to the csu locus together
with chaperone-usher regulators (Bfms and Bfmr) after
addition of 100 µmol/L C6-HSL to culture medium of
A. baumannii ATCC19606, suggesting a strong link
between quorum sensing and forming type I pili.

OmpA and PNAG (encoded by the pga locus) in
A. baumannii play the important roles in the coloniza-
tion, immune evasion, antibiotic resistance and biofilm
formation.44,45 Our results indicated a significant corre-
lation between the expression level of the pgaA gene
and biofilm formation when all of these representative
strains were exposed to either tigecycline or meropenem
(except strain AB05 at sub-MICs of tigecycline). Also,
the transcription level of the ompA gene was positively
correlated with biofilm formation in three strain of
AB10, AB32, and AB55 in the presence of meropenem,
as well as strain of AB10 at sub-MICs of tigecycline.
In support of our findings, Sato et al16 indicated that the
ompA and pgaA expression patterns were positively
correlated with biofilm formation when A. baumannii
strains were exposed to polymyxin B and colistin,
respectively. Also, He et al23 proved that the expression
regulation of the ompA gene was significantly correlated
with forming biofilm at sub-MICs of either levofloxaclin
or meropenem.

Bap plays the important roles in the initial adherence to
abiotic surfaces, the stabilization of mature biofilms, affect-
ing both thickness and biovolume and subsequently the
per sistence in hospital infections.46 Our results showed that the
biofilm formation at the sub-MICs of either levofloxaclin or
cefepime was positively correlated with the expression level
of the bap gene in all of these representative strains.
In agreement with our study, Sato et al16 also, demonstrated
that polymyxin B altered the biofilm formation through the
regulation of the bap gene.
Conclusion
In this study, we indicated that tigecycline rather than meropenem interfered with the induction of biofilm formation in *A. baumannii* strains. Also, the expression level of the *adeB* and *adeG* genes was positively correlated with the transcription level of *abaI* gene, indicating a strong link between the efflux pumps of AdeABC and AdeFGH and quorum sensing. In addition, we confirmed a positive correlation between the transcription level of the *csuE* gene together with its regulatory genes with the biofilm formation in all representative strains in the presence of either meropenem or tigecycline. Hence, blocking the efflux pump by EPIs or regulatory genes of type 1 pili might be an alternative treatment approach to inhibit *A. baumannii* biofilm formation.

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Author contributions
All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

Disclosure
The authors report no conflicts of interest in this work.

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