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ORIGINAL ARTICLE

Different effects of sub-minimum inhibitory concentrations of gentamicin on the expression of genes involved in alginate production and biofilm formation of *Pseudomonas aeruginosa*

Fateme Davarzani, Zahra Yousefpour, Navid Saidi, Parviz Owlia*

Molecular Microbiology Research Center (MMRC), School of Medicine, Shahed University, Tehran, Iran

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ABSTRACT

Background and Objectives: Antibiotics at sub-minimum inhibitory concentrations (sub-MIC) may alter bacterial virulence factors. The objective of this study was to investigate the effect of gentamicin at sub-MIC concentrations on the expression of genes involved in alginate production and biofilm formation of *Pseudomonas aeruginosa*.

Materials and Methods: The broth microdilution method was used to determine the MIC of gentamicin for three *P. aeruginosa* clinical isolates (P1-P3) and standard strains (PAO1 and 8821M). Alginate production and biofilm formation of the bacteria in the presence and absence of sub-MIC concentrations of gentamicin were measured using microtiter plate and carbazole assay, respectively. The real-time PCR method was used to determine the effect of gentamicin at sub-MIC concentrations on the expression level of genes involved in biofilm formation (*pel*A and *psl*A) and alginate production (*alg*D and *alg*U).

Results: Gentamicin at sub-MIC concentrations significantly reduced alginate production, biofilm formation, and the expression of alginate and biofilm-encoding genes in clinical isolate P1. This inhibitory effect was also observed on the alginate production of 8821M strain and biofilm formation of PAO1strain. In clinical isolates, P2 and P3, alginate production, biofilm formation, and the expression of alginate and biofilm-encoding genes were significantly increased in exposure to sub-MIC concentrations of gentamicin.

Conclusion: This study showed that different phenotypic changes in clinical isolates and standard strains of *P. aeruginosa* in exposure to sub-MIC concentrations of gentamicin are associated with changes in the expression of virulence genes. Further researches are required to understand the mechanisms involved in regulating the expression of virulence genes after exposure to sub-MIC concentrations of antibiotics.

Keywords: Alginate; Biofilm; Gene expression; Gentamicin; *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is an important Gram-negative opportunistic pathogen that causes 10-20% of nosocomial infections (1). This organism causes serious and life-threatening infections in individuals with immunological disorders such as patients with cancer, AIDS, and especially cystic fibrosis (2, 3). *P. aeruginosa* has intrinsic resistance to many antimicrobial agents and is also able to expand resistance during antibiotic treatment (4). The ability of *P. aeruginosa* to produce virulence factors and biofilm formation is effective in its pathogenesis (5, 6). Biofilms include surface-associated bacterial communities surrounded by an extracellular matrix (7). Biofilms protect the organisms against antibiot-

*Corresponding author: Parviz Owlia, Ph.D, Molecular Microbiology Research Center (MMRC), School of Medicine, Shahed University, Tehran, Iran. Tel: +98- 21-66952202 Fax: +98-21-66952202 Email: powlia@gmail.com

ics, bacteriophages, disinfectants, and host defense systems (8). Pel, Psl, and alginate are three extracellular polysaccharides produced by *P. aeruginosa* (9). In both mucoid and non-mucoid *P. aeruginosa* strains, Pel and Psl polysaccharides play an essential role in the formation and maintenance of mature biofilm structure (9, 10). *pel*A and *psl*A genes are involved in the synthesis of Pel and Psl polysaccharides, respectively (11). Alginate, which is overproduced in mucoid strains of *P. aeruginosa*, is one of the most important virulence factors of this organism. Alginate is a linear polysaccharide composed of 1-4 linked β-D -mannuronic acid and α-L-guluronic acid (2). Although alginate is not a necessary component for biofilm formation, it can be one of the dominant polysaccharides of the biofilm matrix in mucoid strains and plays a role in the formation of thick, three-dimensional biofilms (12, 13). *alg*D and *alg*U are key genes involved in the regulation and production of alginate (14). Alginate is involved in the development of chronic infections by protecting the organism against the host immune system and neutralizing oxygen radicals (15, 16).

Gentamicin, an aminoglycoside antibiotic, is widely use in the treatment of *P. aeruginosa* chronic infections and is effective at supra-inhibitory concentrations (17, 18). However, bacteria may be exposed to sub-minimum inhibitory concentrations (sub-MIC) of antibiotics during antibiotic therapy (5). Sub-MIC concentrations of antibiotics have been shown to affect bacterial pathogenesis by affecting virulence factors of bacteria (19). However, there is controversy regarding the type of these effects and the relevant mechanism involved (20). Some studies have reported that the bacterial phenotypic changes in exposure to sub-MIC concentrations of antibiotics are due to the expression changes of phenotypes-related genes (5, 18).

In this study, we investigated the effects of sub-MIC concentrations of gentamicin (1/2 MIC and 1/4 MIC) on the expression of genes involved in alginate production (*alg*D and *alg*U) and biofilm formation (*pel*A and *psl*A), the amount of alginate production, and biofilm formation in three clinical isolates of *P. aeruginosa* and standard strains.

MATERIALS AND METHODS

Bacterial isolates. This study was conducted on three clinical isolates of *P. aeruginosa* (P1: from a urine sample, P2 and P3 from sputum samples) collected from Pars and Milad Hospitals in Tehran, and standard strains 8821M and PAO1. The Clinical isolates were identified by standard microbiological tests. The bacteria were kept at -70°C in nutrient broth (NB) (Merck, Germany) with 20% glycerol. The bacteria were cultured on nutrient agar medium (Merck, Germany) and incubated at 37°C for 18-24 hours, before use for each experiment.

Determination of minimum inhibitory concentration (MIC). To determine the MIC of gentamicin Bio Basic Canada-INC) for clinical isolates and standard strains of *P. aeruginosa*, the broth microdilution method was used based on CLSI guidelines (21). The stock solution of gentamicin (5120 µg/ml) was diluted in Mueller-Hinton Broth (MHB) medium (Merck, Germany) to obtain two-fold serial dilutions with the range of 256 µg/ml to 0. 25 µg/ml. Gentamicin dilutions were transferred to microplate wells and inoculated with bacterial suspension with the turbidity of 0.5 McFarland standard. Bacterial culture without gentamicin was used as a positive control, and MHB medium was served as a negative control. The microplate was incubated at 37°C for 24 hours. Then, bacterial growth was investigated in each well, and the minimum concentration of gentamicin which prevented visible growth, was considered as MIC.

Effect of sub-MIC concentrations of gentamicin on the biofilm formation. Evaluation of the biofilm formation of *P. aeruginosa* clinical isolates and standard strain PAO1 was performed using microtiter plate assay (22). The bacterium was cultured in MHB medium at 37°C for 24 hours. Then, 1×10^8 cfu/mL of the bacteria from this culture were inoculated into MHB medium, with sub-MIC concentrations of gentamicin (1/2 MIC and 1/4 MIC). Then, 200 µl of each suspension was transferred in wells of a microplate. Wells containing MHB medium were served as control. The plate was incubated aerobically for 24 hours at 37°C. Then, plate was washed three times with phosphate-buffered saline (PBS). Subsequently, the plate was drained for 5 minutes at room temperature. The biofilm formed in the wells was fixed by 95% ethanol. In the next stage, the biofilm was stained with 150 µl of 1% crystal violet for 10 minutes. The wells were washed with deionized water to remove the excess dye. After drying the plate, the remaining

crystal violet was released and dissolved using 150 µl of acetic acid (33%). The optical density (OD) of each well was measured by 490 nm using a microplate reader (EL×808, BioTek, USA). The assay was repeated three times, and the mean OD values of control wells and test wells were reported. The following formulas were used to classify the biofilm formation (Table 1).

Table 1. Interpretation of optical density (OD) results of biofilm formation (22)

OD	Result
$OD_t \leq OD_c$	Non-biofilm
$OD_c < OD_t < 2\times OD_c$	Weak biofilm
$2\times OD_c < OD_t < 4\times OD_c$	Moderate biofilm
$OD_t \geq 4\times OD_c$	Strong biofilm

OD_c = Mean OD of control wells (MHB without bacteria)
OD_t = Mean OD of test wells (MHB containing antibiotic treated or untreated bacteria)

Effect of sub-MIC concentrations of gentamicin on the alginate production. The amount of alginate produced by *P. aeruginosa* clinical isolates and standard strain 8821M was measured by Carbazole assay with a few modifications as described in previous study (23). From an overnight culture of the bacterium, a suspension was adjusted to 0.5 McFarland standard, and 100 µl of this suspension was added to three culture mediums: MHB medium, MHB with 1/2 MIC concentration of gentamicin, and MHB with 1/4 MIC concentration of gentamicin. The cultures were incubated in shaker incubator at 37°C for 24 hours Then, 140 µl of each bacterial culture was slowly added to 1200 µl of borate-sulfuric acid solution in an ice bath and was vortexed for 4 seconds. Then, 40 µl of 0.2% carbazole solution was added to this mixture and then was immediately vortexed. This suspension was placed at 55°C for 30 min, and then the optical density (OD) was measured at 540 nm by a spectrophotometer device (PerkinElmer, USA). The assay was repeated three times, and the mean OD was calculated.

Effect of sub-MIC concentrations of gentamicin on the expression of genes involved in alginate production and biofilm formation. The effect of sub-MIC concentrations of gentamicin on the expression level of *alg*D, *alg*U, *pel*A, and *psl*A genes was investigated by the real-time PCR. At first, from an overnight culture of the bacterium in MHB medium, a suspension with the turbidity of 0.5 McFarland standard was prepared and inoculated into MHB mediums with sub-MIC concentrations of gentamicin (1/2 MIC and 1/4 MIC). Bacterial culture without gentamicin was used as positive control. Then, these mediums were incubated at 37°C for 24 hours. To prepare the cell pellet, 2 volumes of bacterial culture were added to 1 volume of RNA protect bacteria reagent (Qiagen, Germany) and this mixture was centrifuged at 5000-g for 10 minutes. TE buffer containing 15 mg/ml lysozyme and proteinase K was added to the cell pellet, and the cells were lysed. Total RNA was extracted using RNeasy mini kit (Qiagen, Germany) based on the manufacturer's instructions. RNA samples were analyzed by the NanoDrop device (NanoDrop One, Thermo Fisher, USA) to determine the purity and concentration (24). Conversion of total RNA to cDNA was performed using QuantiTect reverse transcription kit (Qiagen, Germany) following the manufacturer's instructions. Quantitect SYBR Green PCR Kit (Qiagen, Germany) and the Rotor-Gene Q instrument (Qiagen, Germany) were used to carry out the real-time PCR assay. Each real-time PCR reaction was prepared using the manufacturer's instruction. The sequences of the gene-specific primers used for real-time PCR are listed in Table 2. 16S rRNA gene was used as the housekeeping gene to normalize the expression of genes. Based on the sequences of *alg*D, *pel*A, *psl*A, and 16S rRNA genes, which were extracted from the Gen Bank NCBI database, specific primers for each gene were designed using AlellID6 software. The primers used for the *alg*U gene were based on the previous study (25). Each reaction was done in triplicate. At the end of the process, the threshold cycle value (Ct) of each reaction was calculated automatically by the real-time PCR software program. The relative gene expression was calculated with the ΔΔCT method (26).

Statistical analysis. All the tests were performed three times. The analysis of the obtained results was performed with the GraphPad Prism software version 8.0.2, and the mean ± standard deviation (S.D) of the results were reported. The results of the gentamicin-treated bacteria were compared with the control bacteria (untreated) by the one-way ANOVA test. The difference between the groups was considered statistically significant at P-value less than 0.05.

Table 2. Gene-specific primers used for real-time PCR analysis

Gene	Primer Sequence (5′→3′)	Product size	Reference
*alg*D	F: GATGTCTCCAGCACCAAG	164 bp	designed by AlellID6 software
	R: ACGCAGATGAACGATACG		
*alg*U	F: CGATGTGACCGCAGAGGATG	292 bp	25
	R: TCAGGCTTCTCGCAACAAAGG		
*pel*A	F: GTGCTCGCTACCCTCCTC	194 bp	designed by AlellID6 software
	R: CGCTGCTTGGTTCCTTCG		
*psl*A	F: CTGGTCCTGGCGAGCATC	179 bp	designed by AlellID6 software
	R: TTGGCGTCGGTCCTTTCC		
16Sr RNA*	F: TTCGGACCTCACGCTATC	95 bp	designed by AlellID6 software
	R: CCTCTCAGACCAGTTACGG		

*16Sr RNA was used as a housekeeping gene

RESULTS

MIC of gentamicin. MIC values of gentamicin for *P. aeruginosa* clinical isolates P1, P2, P3, standard strains 8821M, and PAO1 were 0.25, 0.25, 1, 2, and 0.5 μg/ml, respectively.

Biofilm formation changes in the presence of sub-MIC concentrations of gentamicin. In this study, the biofilm formed by *P. aeruginosa* clinical isolates and standard strain PAO1 was investigated in the presence and absence of sub-MIC concentrations (1/2 MIC and 1/4 MIC) of gentamicin. In the clinical isolate P1 and standard strain PAO1, which were strong biofilm producers, the biofilm formation was significantly decreased to a weak degree by 1/2 MIC concentration of gentamicin and also significantly decreased to a moderate degree by 1/4 MIC concentration of gentamicin (P <0.001). In the clinical isolates P2 and P3, which were weak biofilm producers, both concentrations of gentamicin could significantly increase the biofilm formation of these isolates to a moderate degree. In these isolates, 1/4 MIC concentration of gentamicin had a greater stimulatory effect on the biofilm formations (P <0.001) (Table 3, Fig. 1).

Alginate production changes in the presence of sub-MIC concentrations of gentamicin. At first, the amount of alginate produced by *P. aeruginosa* clinical isolates and standard strain 8821M was measured in the absence of gentamicin. All the isolates were able to produce the alginate. Then, the effect of sub-MIC concentrations (1/2 MIC and 1/4 MIC) of gentamicin on alginate production was investigated. sub-MIC concentrations of gentamicin could significantly reduce the amount of alginate produced by *P. aeruginosa* 8821M and clinical isolate P1 (P <0.001). The amount of alginate reduction was dependent on

Table 3. The percentage changes of biofilm formation of *P. aeruginosa* clinical isolates and standard strain PAO1 in the presence of sub-MIC concentrations of gentamicin compared to control (absence of gentamicin)

Strain & isolates	1/2MIC GEN	1/4MIC GEN
Standard strain (PAO1)	51% - ↓	37% - ↓
P1	61% - ↓	41% - ↓
P2	39% - ↑	75% - ↑
P3	38% - ↑	70% -↑

↑: Increased biofilm formation compared to control
↓: Decreased biofilm formation compared to control

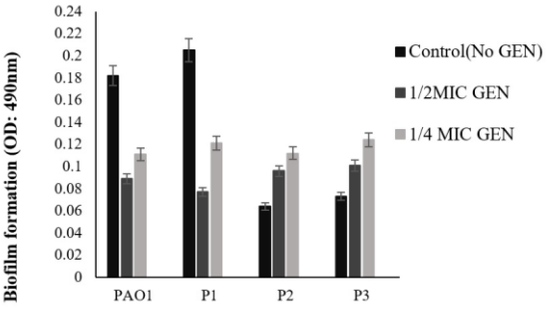

Fig. 1. Biofilm formation of *P. aeruginosa* clinical isolates and standard strain PAO1 in the presence and absence of sub-MIC concentrations of gentamicin

the concentration of gentamicin. Gentamicin at 1/2 MIC concentration had a greater decreasing effect on the alginate production than 1/4MIC concentration. The amount of alginate produced by clinical isolates P2 and P3 was significantly increased in exposure to sub-MIC concentrations of gentamicin (P <0.001). In these isolates, the stimulatory effect of gentamicin on the alginate production in the presence of 1/4 MIC concentration of gentamicin was higher than 1/2 MIC (Table 4, Fig. 2).

Table 4. The percentage changes of alginate production of *P. aeruginosa* clinical isolates and standard strain 8821M in the presence of sub-MIC concentrations of gentamicin compared to control (absence of gentamicin)

Strain & isolates	1/2 MIC GEN	1/4 MIC GEN
Standard strain (8821M)	47% - ↓	8% - ↓
P1	65% - ↓	61% - ↓
P2	35% - ↑	57% - ↑
P3	51% - ↑	93% - ↑

↓: Decreased alginate production compared to control
↑: Increased alginate production compared to control

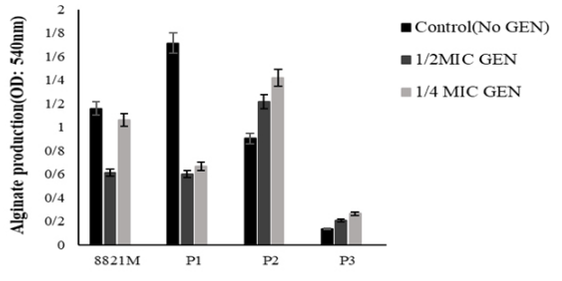

Fig. 2. Alginate production of *P. aeruginosa* clinical isolates and standard strain 8821M in the presence and absence of sub-MIC concentrations of gentamicin

Expression changes of genes involved in alginate production and biofilm formation in the presence of sub-MIC concentrations of gentamicin. The expression level of alginate and biofilm-encoding genes in clinical isolates and standard strains of *P. aeruginosa* that had been treated with sub-MIC concentrations of gentamicin (1/2 MIC and 1/4 MIC) were compared with the control samples (untreated) using the real-time PCR process. The fold changes of genes expression are presented in Table 5. Gentamicin at sub-MIC concentrations reduced the expression of genes involved in biofilm formation (*pel*A and *psl*A), and alginate production (*alg*D and *alg*U) in *P. aeruginosa* standard strains PAO1 and 8821M, respectively. Reducing the expression of alginate and biofilm-encoding genes was also observed in *P. aeruginosa* clinical isolate P1. In these standard strains and clinical isolate, 1/2 MIC concentration of gentamicin had a greater inhibitory effect on the expression of all genes than 1/4 MIC concentration of gentamicin. sub-MIC concentrations of gentamicin increased the expression of alginate and biofilm-encoding genes in *P. aeruginosa* clinical isolates P2 and P3. In these isolates, the expression of the genes in the presence of 1/4MIC concentration of gentamicin was higher than 1/2 MIC concentration of gentamicin. In all clinical isolates and standard strains studied, there was a correlation between the effects of sub-MIC concentrations of gentamicin on the expression of genes involved in alginate production and biofilm formation with the effects of these concentrations on alginate and biofilm production. According to these results, it is speculated that changes in the expression of alginate and biofilm-encoding genes are responsible for the changes in the amount of alginate and biofilm production in exposure to sub-MIC concentrations of gentamicin (Fig. 3).

Table 5. Expression fold changes of genes involved in alginate production and biofilm formation of *P. aeruginosa* clinical isolates and standard strains in the presence of sub-MIC concentrations of gentamicin compared to control (NO GEN).

Strains & isolates	Genes	Expression fold change	
		1/2 MIC GEN	1/4 MIC GEN
8821M	*alg*D	20 fold - decrease	1.31 fold - decrease
	*alg*U	14.28 fold - decrease	12.5 fold - decrease
PAO1	*pel*A	4 fold- decrease	2.5 fold - decrease
	*psl*A	10 fold- decrease	4 fold - decrease
P1	*alg*D	6.94 fold - decrease	2.46 fold - decrease
	*alg*U	12 fold - decrease	8 fold - decrease
	*pel*A	8.3 fold - decrease	3.7 fold - decrease
	*psl*A	2.5 fold - decrease	1.33 fold - decrease
P2	*alg*D	1.287fold - increase	22.83 fold - increase
	*alg*U	2.62 fold - increase	5.29 fold - increase
	*pel*A	3.66 fold - increase	18.57 fold - increase
	*psl*A	3.31 fold - increase	20.53 fold - increase
P3	*alg*D	3.48 fold - increase	5.3 fold - increase
	*alg*U	2.71 fold - increase	3.41 fold - increase
	*pel*A	8 fold - increase	10.41 fold - increase
	*psl*A	9.44 fold - increase	15.19 fold - increase

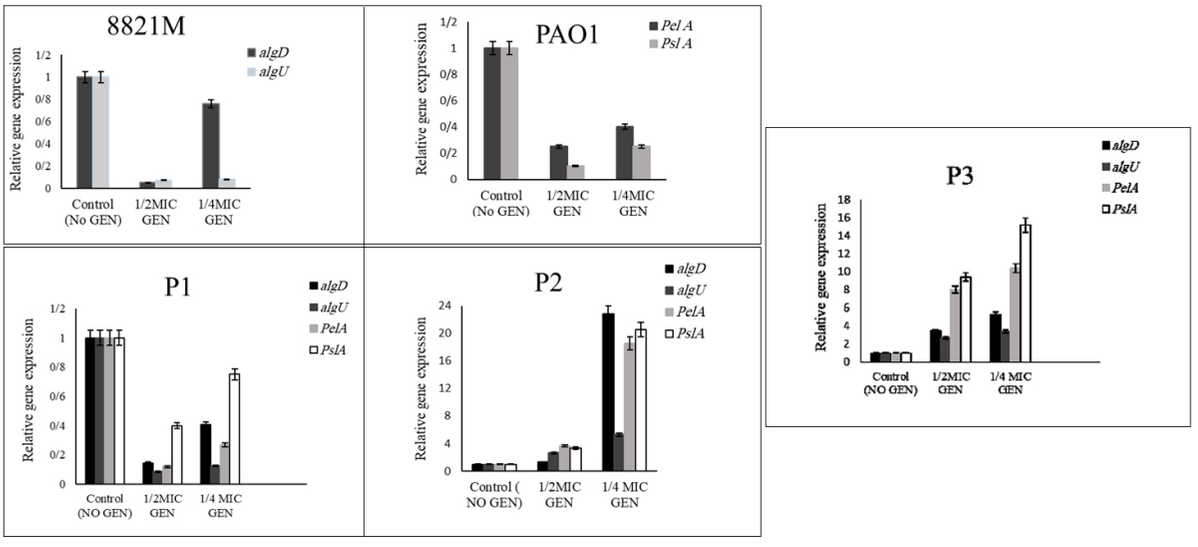

Fig. 3. Effect of sub-MIC concentrations of gentamicin on the expression of *alg*D, *alg*U, *pel*A, and *psl*A genes in *P. aeruginosa* standard strains and clinical isolates

DISCUSSION

P. aeruginosa is one of the most important human opportunistic pathogens, which causes major problems in individuals with immunological disorders due to acute and chronic infections. (27). Alginate production and biofilm formation play an important role in the development of chronic *P. aeruginosa* infections by protecting the organism against the host immune system (9). It is difficult to treat and manage these infections due to the innate resistance of this organism to many antimicrobial agents and the rapid spread of resistance during antimicrobial therapy (4). Some studies attributed the mechanism of antibiotic resistance to effects of sub-MIC concentrations of antibiotics on mutation rates and horizontal gene transfer. Also, exposure to sub-MIC concentrations of antibiotics has been shown to affect the expression of bacterial virulence genes and bacterial physicochemical characteristics (28). There are contradictory reports on the effects of sub-MIC concentrations of antibiotics on pathogenicity of *P. aeruginosa*. Several studies have shown that some antibiotics such as ceftazidime, piperacillin-tazobactam, and gentamicin at sub-MIC concentrations have an inhibitory effect on the virulence factors of *P. aeruginosa* (9, 19). However, a number of other studies have shown the stimulatory effect of sub-MIC concentrations of some antibiotics such as vancomycin, tetracycline, azithromycin, and ampicillin on the virulence factors of *P. aeruginosa* (5, 20). This study examined the effect of sub-MIC concentrations of gentamicin on the expression of genes involved in alginate production and biofilm formation, as well as the amount of alginate and biofilm production in three clinical isolates and standard strains of *P. aeruginosa*. In the current study, gentamicin at the sub-MIC concentration reduced the expression of genes involved in alginate production (*alg*D, *alg*U) and biofilm formation (*pel*A, *psl*A) in *P. aeruginosa* 8821M and PAO1, respectively. In addition, these concentrations significantly decreased the amount of alginate production of *P. aeruginosa* 8821M and biofilm formation of *P. aeruginosa* PAO1. The inhibitory effect of sub-MIC concentration of gentamicin on alginate production, biofilm formation, and expression of their related genes was also observed in the *P. aeruginosa* clinical isolate P1. These results are consistent with the results of some studies. For example, Otani et al. showed that 1/4 MIC concentration of ceftazidime could reduce the expressions of *pel*A and *psl*A genes and subsequently biofilm volume in *P. aeruginosa* PAO1 (9). In a study, Bahari et al. demonstrated the inhibitory effects of sub-MIC concentrations of curcumin, azithromycin, and gentamicin on the expression of regulatory genes of the quorum-sensing (QS) system, QS signal molecules, motility, and biofilm formation of *P. aeruginosa* PAO1. They concluded that sub-MIC concentrations of antibiotics alter bacterial virulence factors by affecting the QS system (29). In a study, Gupta et al. attributed the mechanism of reduction in the amount of biofilm forma-

tion, production of alginate, rhamnolipid, elastase, siderophore, and protease in four clinical isolates of *P. aeruginosa* and standard strain PAO1 in the presence of sub-MIC concentrations of ciprofloxacin to a reduction in QS signal molecules. (30).

On the other hand, sub-MIC concentrations of gentamicin had a stimulatory effect on the virulence factors of both *P. aeruginosa* clinical isolates P2 and P3 and significantly increased the expression of *alg*D, *alg*U, *pel*A, and *psl*A genes, alginate production, and biofilm formation. The stimulatory effect of sub-MIC concentrations of some antibiotics on bacterial virulence factors has been demonstrated in a number of studies. For example, in a study, Bagge et al. showed an increase in the expression of alginate genes (*alg*D, *alg*U), level of alginate production, and biofilm volume of *P. aeruginosa* PAO1 by imipenem at sub-MIC concentrations (31). Shen et al. revealed that sub-MIC concentrations of vancomycin, tetracycline, azithromycin, and ampicillin increased the expression of some virulence genes such as *phz*A1, *phz*A2, *rhl*AB, as well as rhamnolipid and pyocyanin production in *P. aeruginosa* PAO1 (5).

The present study showed that sub-MIC concentrations of gentamicin have different effects on the expression of genes involved in alginate production and biofilm formation in clinical isolates and standard strains of *P. aeruginosa* that cause different changes in the amount of alginate production and biofilm formation. These results are similar to the results of a study conducted by Navidifar et al. which showed different effects of sub-MIC concentrations of meropenem (inhibitory and stimulatory) on the expression levels of biofilm-encoding genes and the amount of biofilm formation in different strains of *A. baumannii* (32). The reason for the different effects of sub-MIC concentrations of gentamicin on the gene expression of different clinical isolates is not clear to the authors of the present study. The bacterial responses to the presence of sub-MIC concentrations of antibiotics seems to depend on the type of bacterial isolates and strains and these responses are specific and defensive (17). Therefore, it is also important to use molecular typing methods before choosing an antibiotic treatment. It has been suggested that antibiotics are involved in signaling mechanisms. They act as signaling molecules at sub-MIC concentrations that regulate and modulate the expression of coding genes of virulence factors, but the mechanism and extent of this regulation is not well understood (5). Some recent reports have shown that the mechanism of the effect of sub-MIC concentrations of antibiotics on bacterial virulence factors is related to the effect of these concentrations on the QS signal molecules and the genes associated with this system (29, 30). Since the expression of virulence factors and biofilm formation in *P. aeruginosa* is regulated by the QS system, sub-MIC concentrations of gentamicin may react with this system, leading to a change in the expression of genes involved in alginate production and biofilm formation. Therefore, further studies should be performed to examine the effect of sub-MIC concentrations of gentamicin on the QS system and its association with the change of virulence factors of *P. aeruginosa*.

In this study, sub-MIC concentrations of gentamicin showed both inhibitory and stimulatory effects on the biofilm formation and alginate production of various clinical isolates of *P. aeruginosa*. Overexpression of alginate and biofilm-encoding genes, which subsequently affect their production, is clinically important because the pathogenicity may be subsequently increased. Furthermore, increasing the amount of alginate and biofilm production reduces the antibacterial efficacy of antibiotics. Therefore, the effects of gentamicin therapy may be compromised. It has been suggested that this situation may be prevented by using suitable combinations of antibiotics (28). The animal tests should be performed to investigate the effects of sub-MIC concentrations of gentamicin on the pathogenesis of *P. aeruginosa*, because this study was conducted *in vitro* and the bacterial response to the presence of antibiotics *in vivo*, in addition to the antibiotic concentration was influenced by various factors such as the status of the host immune system, age, etc. In addition, it is necessary to study the main molecules involved in bacterial responses to sub-MIC concentrations of antibiotics. To increase the effectiveness of antibiotic therapy, it is needed to discover new treatment strategies that target these molecules.

CONCLUSION

The present study demonstrated that sub-MIC concentrations of gentamicin could induce different changes in the expression of virulence genes and consequently phenotypes associated with these genes in distinct clinical isolates and standard strains

of *P. aeruginosa*. Further and broader studies are required to understand the complex regulation of virulence genes expression in exposure to sub-MIC concentrations of antibiotics. Particularly, a greater number of different clinical isolates should be studied.

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