Original Article

Effects of Sub-Minimum Inhibitory Concentrations of Gentamicin on Alginate Produced by Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract

Background: Bacterial virulence factors may be influenced by sub-minimum inhibitory concentrations (sub-MICs) of antibiotics. The main purpose of this study was to investigate the effects of gentamicin at sub-MICs (0.5 MIC and 0.25 MIC) on alginate production of clinical isolates of *P*seudomonas *aeruginosa*.

Materials and Methods: The minimum inhibitory concentrations of gentamicin against 88 clinical isolates of *P. aeruginosa* were determined using the broth microdilution method. Alginate production of the isolates in the absence and presence of gentamicin at sub-MICs was assessed by the carbazole method. The presence of alginate in clinical isolates was confirmed by the detection of alginate genes (*algD* and *algU*) using the PCR method.

Results: All the isolates had the ability of alginate production and were positive for *algD* and *algU* genes. sub-MICs of gentamicin significantly increased alginate production of 34 isolates (38.6%). On the other hand, in 49 isolates (55.7%), alginate production was significantly increased after treatment with sub-MICs of gentamicin. In five isolates (5.7%), the alginate production was reduced in exposure to 0.5 MIC of gentamicin while it was increased by gentamicin at 0.25 MIC.

Conclusion: This study showed different effects of gentamicin at sub-MICs on the alginate production of clinical isolates of *P*. *aeruginosa*. Further research is highly recommended to understand the mechanism of different responses of *P*. *aeruginosa* isolates to the exposure of sub-MICs of gentamicin.

Keywords: Alginates, gentamicins, *Pseudomonas aeruginosa*, virulence factors

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Introduction

Pseudomonas aeruginosa is a human opportunistic pathogen that causes different infections including respiratory infections, urinary tract infections, wound infections, burn infections, and septicemia in patients with primary and acquired immunodeficiency, particularly cystic fibrosis patients.[1,2] Increasing the number of infections caused by *P. aeruginosa* is one of the world's health concerns because of its intrinsic resistance to many antibiotics and also the ability to acquire and develop resistance during treatment.[3] *P*. *aeruginosa* produces several virulence factors and toxins that are involved in the invasion and toxicity of this bacterium.[4] Alginate is the main virulence factor of *P. aeruginosa* mucoid strains associated with the development of the chronic phase of infections, especially respiratory chronic infections in patients with cystic fibrosis.[5,6] Alginate, an extracellular polysaccharide, is composed of β-d-mannuronic acid and α-l-guluronic acid

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monomers, which are linked by β1 → 4 glycosidic linkages.[7] *algD* and *algU* genes are among the main genes responsible for alginate biosynthesis.[8] Alginate forms a polyanionic barrier protecting bacteria against the host immune system and penetration of certain antibiotics.[9,10] According to the results of some reports, alginate increases the resistance of *P. aeruginosa* to specific antibiotics.[11] In *P. aeruginosa* mucoid strains, alginate is the major exopolysaccharide of biofilm structure and is involved in the formation of differentiated biofilms (thick three-dimensional), which are highly resistant to antibiotic treatment.[12] Any process leading to changes in the alginate production level affects the pathogenesis of *P. aeruginosa.*

Antibiotics are the main agents to treat bacterial infections, which should be prescribed at supra-inhibitory concentrations and for successive doses. However, *in vivo*, the administered concentrations of antibiotics may become sub-minimum inhibitory concentrations (sub-MICs) after a specific period following a dose.[13] It has been demonstrated that antibiotics even at their sub-MICs can affect bacterial pathogenesis through different mechanisms such as altering virulence factors of bacteria.[14] The kind of these effects depends on various factors and is a controversial subject. sub-MICs of antibiotics have been reported to induce change in the level of bacterial virulence gene expression, which lead to different phenotypes. Also, it has been shown that antibiotics at sub-MICs can be directly responsible for emerging of new antibiotic resistance by elevating mutation rate and spreading of mutation through horizontal gene transfer.[15] There are several studies indicating that *P. aeruginosa* virulence factors are influenced by sub-MICs of antibiotics.[16] The principal objective of this research was to determine the effects of gentamicin at sub-MICs (0.5 MIC and 0.25 MIC) on the alginate production of different clinical isolates of *P. aeruginosa.*

Materials and Methods

Bacterial isolates

Eighty-eight clinical isolates of *P. aeruginosa* were selected for this study. The isolates were isolated from different clinical specimens (blood, urine, sputum, wound, and tracheal) in selected hospitals in Tehran between 2017 and 2018. Standard microbiological tests were performed to identify the *P. aeruginosa* clinical isolates. These tests included colony morphology, Gram staining, culture on TSI medium, catalase test, oxidase test, oxidation-fermentation test (OF), and growth at 42°C. Also, two *P*. *aeruginosa* standard strains were served in this study includin: *P. aeruginosa* ATCC27853 for quality control in MIC determination test and *P*. *aeruginosa* 8821 M as a positive control for alginate production assay. The bacteria were stored at −70°C in nutrient broth (Merck, Germany) containing 20% glycerol. This project was approved by the research ethics committee of Shahed University with approval ID IR.SHAHED.REC.1398.73.

Determination of Minimum Inhibitory Concentration (MIC) of gentamicin

Determination of MIC values of gentamicin (Bio Basic Canada-INC) against clinical isolates of *P. aeruginosa* and standard strain 8821M was done using the broth microdilution method based on the Clinical and Laboratory Standard Institute (CLSI) protocols.[17] At first, a stock solution of gentamicin with a final concentration of 5120 μg/mL was prepared. Then, the dual consecutive concentrations of gentamicin (2048 -0. 25 μg/mL) were prepared from the stock solution by diluting it with Mueller–Hinton Broth (MHB) medium (Merck, Germany). Each of the gentamicin concentrations was shifted to wells of a microplate, and inoculated with bacterial suspension with a concentration of 1×10^6 CFU/mL. For each isolate, a positive control (antibiotic-free culture) and negative control (bacterial-free culture) were considered, separately. The microplate was placed at 37°C for 24 h. The lowest level of gentamicin without seeable turbidity was reported as MIC. To determine the resistance pattern of the isolates to gentamicin, the MIC results were interpreted according to the CLSI guideline (MIC ≤4 μg/mL: sensitive, MIC ≥16 μg/mL: resistant).

Effect of sub-MICs of gentamicin on the alginate production

Evaluation of alginate production of bacteria treated and untreated with sub-MICs of gentamicin (0.5 MIC and 0.25 MIC) was performed using Carbazole assay according to previous study.[18] At first, *P. aeruginosa* isolate was cultured on the nutrient agar medium (Merck, Germany) overnight at 37°C. Then, from this culture, 1×10^8 CFU/mL of the bacteria were added to the MHB medium and MHB mediums containing sub-MICs of gentamicin and then were incubated in an incubator shaker at 37˚C. MHB was used as a negative control. After 24 h, 140 µL of each culture was mixed with 1200 µL of borate-sulfuric acid solution, which had been placed in an ice bath. The mixture was vortexed and was returned to the ice bath, and then 40 µL of 0.2% carbazole solution was added to it. After vortexing, the mixture was incubated at 55°C for 30 min, and then optical density (OD) was read at 540 nm by a spectrophotometer (PerkinElmer, USA). All the steps were run in triplicate.

Standard curve of alginate

Alginate standard curve was plotted to express the concentration of alginate produced by *P. aeruginosa* clinical isolates as micrograms per microliter. At first, 1 mg of alginic acid (Sigma Aldrich, USA) was dissolved in 10 mL distilled water to prepare an alginate stock solution with a concentration of 1 mg/mL. The stock solution was diluted in distilled water and 16 dilutions were prepared with the range of 20–320 µg/mL. Carbazole assay was performed for all the dilutions. The OD values of alginate standard solutions were placed against their corresponding concentrations on a graph and the standard curve was plotted.

Molecular detection of alginate genes

The presence of alginate in *P. aeruginosa* clinical isolates was confirmed by the detection of important genes involved in the biosynthesis of alginate (*algD* and *algU*) using the PCR method. Table 1 shows the sequences of used primers. Specific primers for *algD* gene were designed by AlellID6 software. Based on the extracted sequences of this gene from the Gen Bank NCBI database. The sequences of primers used for the *algU* gene were according to the previous study.[19] *P. aeruginosa* 8821M was used as a positive control. The total volume of PCR reaction was 25 µL. The components of each PCR reaction included: 12.5 µL of Taq DNA Polymerase Master Mix (Ampliqon, Denmark), 0.5 µL of each primer, 6.5 µL of ddH_2O, and 5 µL of Template DNA. The boiling method was used for the extraction of DNA from studied isolates. Amplification of the genes was performed in thermocycler instrument (Bio-Rad, USA) as follows: Initial denaturation at 94°C for 5 min and 34 cycles included denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72 ºC for 1 min and at the end, final extension at 72°C for 10 min. For separation of PCR products, electrophoresis was carried out on a 2% agarose gel which had been stained with a safe stain (Sinacolon, Iran).

Statistical analysis

Alginate production assay was performed in triplicate and obtained results were analyzed with GraphPad Prism software version 8.0.2. Results were reported as the means ± standard deviation (SD). Determination of the statistical significance of differences between gentamicin-treated bacteria and control bacteria (untreated) was performed using the one-way ANOVA test. Pearson correlation was used to determine the relationship between the studied factors. *P* values less than 0.05 were considered as the significance level.

Results

MIC results

The MICs of gentamicin against *P. aeruginosa* isolates, which were obtained by broth microdilution method, were interpreted based on CLSI protocols. Sixty-two isolates (70%) were susceptible to gentamicin and MIC values were between 0.25 and 2 µg/mL. The range of MIC values in 26 clinical isolates (30%) was between 16 and 512 µg/mL and therefore were considered as resistant isolates [Figure 1].

Table 1: Sequences of primers used for molecular detection of *algD* and *algU* genes

Gene	Primer Sequence (5′→3′)	Product size
algD	Forward: 5’- GATGTCTCCAGCACCAAG-3’	
algU	Reverse: 5’- ACGCAGATGAACGATACG-3’	164 bp
	Forward: 5’- CGATGTGACCGCAGAGGATG -3’	292 bp
	Reverse: 5’- TCAGGCTTCTCGCAACAAAGG-3’	

Standard curve of alginate

Standard curve of alginate was made by plotting the concentrations of alginate standard solutions on the X-axis and their OD values on the Y-axis. This graph was linear which indicated that the OD of each solution was proportional to its concentration [Figure 2]. Therefore, this curve was used to obtain the concentration of alginate produced by *P. aeruginosa* clinical isolates.

Effects of sub-MICs of gentamicin on alginate production

The amount of alginate produced by bacteria untreated and treated with sub-MICs of gentamicin (0.5 MIC and 0.25 MIC) was measured. The ability of alginate production was observed in all the studied *P. aeruginosa* isolates. The concentration of alginate among these isolates was in the range of 4–312 µg/mL. The level of alginate production of the isolates in exposure to 0.5 MIC of gentamicin was between 0 and 349 µg/mL and in exposure to 0.25 MIC of gentamicin was in the range of 6–343 µg/mL. *P. aeruginosa* clinical isolates showed different responses to the exposure of sub-MICs of gentamicin. In 34 clinical isolates (38.6%) and *P. aeruginosa* 8821 M, alginate production was significantly reduced after treatment with sub-MICs of gentamicin ($P < 0.001$). This inhibitory effect was concentration-dependent and 0.5 MIC of gentamicin had a more inhibitory effect on alginate production relative to 0.25 MIC of gentamicin. On the other hand, sub-MICs of gentamicin could significantly increase the alginate production of 49 clinical isolates (55.7%) ($P < 0.001$). In these isolates, 0.25 MIC of gentamicin had a more stimulatory effect on alginate production. In five clinical isolates (5.7%), the alginate production was decreased in exposure to 0.5 MIC of gentamicin while it was increased by 0.25 MIC of gentamicin [Figure 3].

In all the studied isolates, we did not find any significant correlation between the amount of alginate production and the type of its change after treatment sub-MICs of gentamicin ($P > 0.05$).

Also, there was not any significant relationship between the alginate production change of each isolate and resistance pattern to gentamicin ($P > 0.05$).

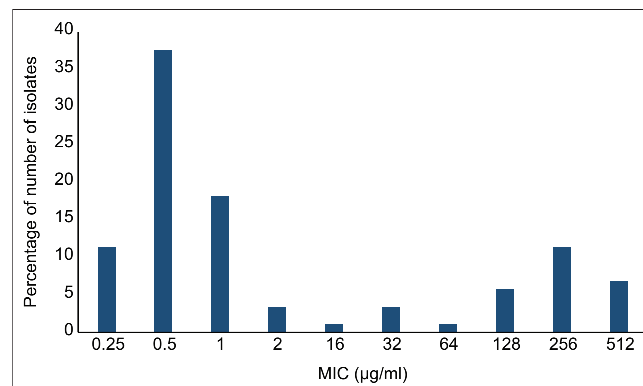

Figure 1: Frequency of MICs of gentamicin against *P. aeruginosa* isolates

Molecular detection of alginate genes

Alginate coding genes (*algD* and *algU*) were identified using the PCR method to confirm the presence of alginate in clinical isolates of *P. aeruginosa*. *algD* and *algU* genes were detected in all the studied isolates, so the frequency of these genes was reported 100% [Figure 4].

Discussion

Pseudomonas aeruginosa is an invasive opportunistic organism responsible for different types of acute and chronic infections in patients with immunodeficiency disorders.[4] One of the effective mechanisms involved in the survival of *P. aeruginosa* in the lungs of patients with respiratory infections is the overproduction of an exopolysaccharide called alginate by this bacterium. Alginate production has been shown to be associated with the pathogenicity of chronic infections of *P. aeruginosa*, which causes a significant increase in morbidity and mortality.[20,21] Thus, the inhibition of alginate production is essential to control and prevent infections caused by *P. aeruginosa* mucoid strains.

In the course of antibiotic therapy, bacteria may be encountered by sub-MICs of antibiotics due to several reasons including restricted drug accessibility, prophylactic treatments with low doses of antibiotics, incomplete antibiotic treatment, etc.[15] It has been demonstrated that the production of bacterial virulence factors may be influenced by antibiotics at sub-MICs. Antibiotics at sub-MICs have been demonstrated to have both stimulatory and inhibitory effects on bacteria such as *P. aeruginosa*.[16] The present study was accomplished to examine the effects of gentamicin at sub-MICs (0.5 MIC and 0.25 MIC) on the alginate production of 88 clinical isolates of *P. aeruginosa.* All the studied isolates were observed to be alginate producers and the frequency of alginate coding genes (*algD* and *algU*) among these isolates was 100%. Some other studies have been carried out on the frequency of alginate production and alginate coding genes among *P. aeruginosa* isolates. For instance, in a study conducted by Ghadaksaz *et al*.[19] on 104 clinical isolates, 89.4% of the isolates could produce alginate, and the prevalence of *algD* and *algU* genes was 87.5% and 90.4%, respectively. In a study, Pournajaf *et al*.[22] reported that among 143 isolates of *P. aeruginosa*, 88.8% of the isolates were alginate producers, and 92.3% and 86.7% of the isolates were positive for *algD* and *algU* genes, respectively.

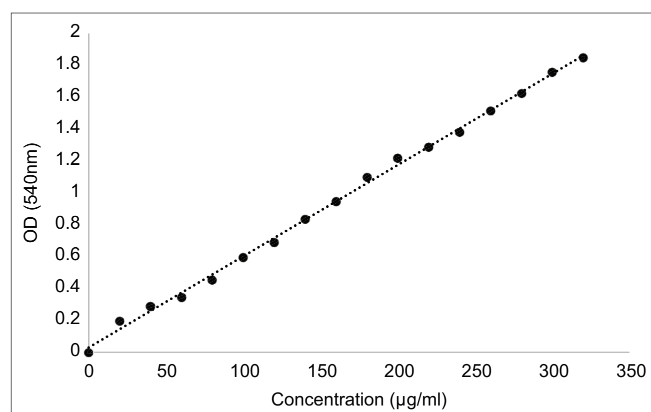

Figure 2: Standard curve of alginate

In the present study, treatment of clinical isolates of *P. aeruginosa* with sub-MICs of gentamicin resulted in different changes in the alginate level of the studied isolates. Gentamicin at sub-MICs significantly decreased the alginate level by 38/6% of the isolates. The inhibitory effect of antibiotics at sub-MICs has been reported in some studies. In a study, Khan *et al*.[23] revealed that aminoglycoside antibiotics (gentamicin, tobramycin, amikacin, etc) could inhibit biofilm formation, bacterial motility, the production of LasA protease enzymes, pyocyanin, and pyoverdine in *P. aeruginosa* PAO1. In a study conducted by Srivastava *et al*.[24] sub-MICs of meropenem and ciprofloxacin reduced biofilm formation in two extensive drug-resistant (XDR) isolates of *P. aeruginosa* isolated from diabetic foot ulcers.

On the other hand, a significant increase was observed in the amount of alginate production of 55.7% of the isolates in exposure to sub-MICs of gentamicin. These results are in agreement with some studies in which the stimulatory effect of antibiotics at sub-MICs on bacteria has been shown. In a study by Hagras *et al*.[25] sub-MICs of cefepime increased the expression of biofilm-associated genes and subsequently biofilm formation in six clinical isolates of *P. aeruginosa*. Linares *et al*.,[26] examined the effects of some antibiotics classes at sub-MICs on some factors of *P. aeruginosa* PAO1. In their study, tobramycin, ciprofloxacin, and tetracycline induced biofilm formation, tobramycin increased swarming and swimming motility, and tetracycline increased genes expression of type III secretion system and subsequently bacterial cytotoxicity.

In the current study, in 5.7% of the clinical isolates, the effect of 0.5 MIC of gentamicin on alginate production differed

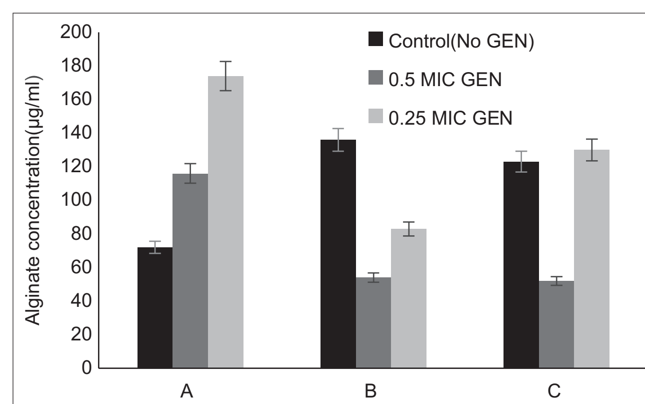

Figure 3: Effects of sub-MICs of gentamicin on alginate production of clinical isolates of *P. aeruginosa.* (A) stimulatory effect, (B) inhibitory effect, (C) inhibitory effect at 0.5 MIC and stimulatory effect at 0.25 MIC concentration of gentamicin. The graph indicates average concentration of alginate produced by clinical isolates

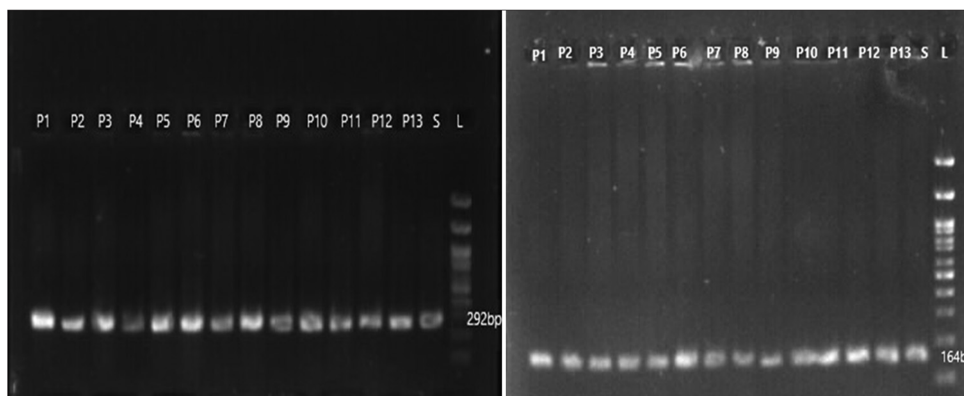

Figure 4: Electrophoresis of PCR products of *algU* (292bp) and *algD* (164bp) genes. L: 1Kb Ladder, P1-P13: clinical isolates, S: Positive control (*P. aeruginosa* 8821 M)

from the effect of 0.25 MIC of gentamicin. In these isolates, alginate production was inhibited following exposure to 0.5 MIC of gentamicin while it was stimulated by 0.25 MIC of gentamicin. Similar to these results, Navidifar *et al*.[27] showed that biofilm formation of an *Acinetobacter baumannii* clinical strain was inhibited by meropenem at 0.5 MIC while it was induced at 0.125 MIC of meropenem.

In the present study, gentamicin at sub-MICs showed different effects on the alginate production of studied clinical isolates. In consistent with these results, Yousefpour *et al*.[28] showed different effects of sub-MICs of gentamicin (0.5 MIC and 0.25 MIC) on biofilm formation of 100 clinical isolates of *P. aeruginosa*. In their study, gentamicin at sub-MICs reduced the capacity to form biofilm in 31% of isolates, whereas increased it in 46% of isolates. Also, in 3% of clinical isolate, gentamicin at 0.5 MIC resulted in a significant decrease in biofilm formation while at 0.25 MIC significantly increased biofilm formation. The mechanisms in charge of different responses of distinct *P. aeruginosa* isolates to the exposure sub-MICs of gentamicin are not explicit to the authors of the current study. Some research ascribed the mechanisms involved in bacterial phenotypic changes caused by sub-MICs of antibiotics to the changes in the expression level of virulence genes. For example, Bagge *et al*.[29] indicated that changes in the expression level of alginate-related genes (*alg*D and *alg*U) in the presence of sub-MICs of imipenem resulted in a change in the alginate level and the capacity of biofilm formation of *P. aeruginosa* PAO1. There is a hypothesis saying that antibiotics at sub-MICs can be as signaling molecules and the expression of virulence-related genes seems to be regulated and modulated by sub-MICs of antibiotics.[14] On the other hand, since the virulence gene expression and production of virulence factors are controlled by the quorum-sensing (QS) system, some studies have reported that expression changes of virulence-related genes in bacteria treated with sub-MICs of antibiotics, which subsequently affect the production of virulence factors, are due to the effects of these concentrations on QS signal molecules and QS-related genes. For example, Roudashti *et al*.[30] reported that curcumin, ceftazidime, and ciprofloxacin at sub-MICs inhibited motility and biofilm formation of *P. aeruginosa* PAO1 by repressing the expression of QS-related genes and QS signal molecules. Thus, further studies are required to evaluate whether or not sub-MICs of gentamicin affect the QS system and also the expression level of genes involved in alginate production.

In this study, the effects of sub-MICs of gentamicin on different clinical isolates of *P. aeruginosa* included both stimulatory and inhibitory effects. It is speculated that bacterial responses following exposure to sub-MICs of antibiotics are dependent on the isolates and strains type and also the type of used antibiotics. Overproduction of alginate caused by sub-MICs of gentamicin can lead to the persistence of bacteria in chronic infections and subsequently antimicrobial resistance may be developed. According to the probable unfavorable effects of antibiotics at sub-MICs on some bacterial isolates, it is suggested that antibiotics should be consumed in an appropriate dosage to avoid bacterial exposure to sub-MIC levels of antibiotics. The results reported in this study are *in vitro* data. *In vivo*, the pathogenesis of *P. aeruginosa* is affected by a number of different factors including host cells, presence of other bacteria, availability of different nutrients, other stresses, etc. Thus, *in vivo* studies such as animal tests should be conducted to confirm the effect of sub-MICs of gentamicin on bacterial pathogenesis. In addition, performing further studies are needed to determine molecular mechanisms responsible for changes in bacterial virulence factors at sub-MICs of antibiotics. Discovering and developing therapeutic agents which target the main molecular factors involved in bacterial responses to sub-MICs of antibiotics, can increase the effectiveness of antibiotic therapy.

Conclusion

The present study indicated that the different responses of clinical isolates of *P. aeruginosa* to the presence of sub-MICs of gentamicin resulted in different changes in alginate levels. Further research should be carried out to determine the mechanisms involved in these responses and discover new treatment strategies based on these mechanisms for the prevention and treatment of *P. aeruginosa* infections.

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Conflicts of interest

There are no conflicts of interest.

References

1. Skariyachan S, Sridhar VS, Packirisamy S, Kumargowda ST, Challapilli SB. Recent perspectives on the molecular basis of biofilm formation by *Pseudomonas aeruginosa* and approaches for treatment and biofilm dispersal. Folia Microbiol 2018;63:413-32.
2. Ghadam P, Akhlaghi F, Ali AA. One-step purification and characterization of alginate lyase from a clinical *Pseudomonas aeruginosa* with destructive activity on bacterial biofilm. Iran J Basic Med Sci 2017;20:467-73.
3. Kaszab E, Radó J, Kriszt B, Pászti J, Lesinszki V, Szabó A, *et al*. Groundwater, soil and compost, as possible sources of virulent and antibiotic-resistant *Pseudomonas aeruginosa*. Int J Environ Health Res 2021;31:848-60.
4. Tuon FF, Dantas LR, Suss PH, Tasca Ribeiro VS. Pathogenesis of the *pseudomonas aeruginosa* biofilm: A review. Pathogens 2022;11:300.
5. Otani S, Hiramatsu K, Hashinaga K, Komiya K, Umeki K, Kishi K, *et al*. Sub-minimum inhibitory concentrations of ceftazidime inhibit *Pseudomonas aeruginosa* biofilm formation. J Infect Chemother 2018;24:428-33.
6. Hao M, Ma W, Dong X, Li X, Cheng F, Wang Y. Comparative genome analysis of multidrug-resistant *Pseudomonas aeruginosa* JNQH-PA57, a clinically isolated mucoid strain with comprehensive carbapenem resistance mechanisms. BMC Microbiol 2021;21:133.
7. Kamali E, Jamali A, Ardebili A. Evaluation of antimicrobial resistance, biofilm forming potential, and the presence of biofilm-related genes among clinical isolates of *Pseudomonas aeruginosa*. BMC Res Notes 2020;13:27.
8. Ellappan K, Belgode Narasimha H, Kumar S. Coexistence of multidrug resistance mechanisms and virulence genes in carbapenem-resistant *Pseudomonas aeruginosa* strains from a tertiary care hospital in South India. J Glob Antimicrob Resist 2018;12:37-43.
9. Bieber Urbauer RJ, Gilmore JM, Rosasco SE, Hattle JM, Cowley AB, Urbauer JL. Cloning, high yield overexpression, purification, and characterization of AlgH, a regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. Protein Expr Purif G2005;43:57-64.
10. Moradali MF, Donati I, Sims IM, Ghods S, Rehm BH. Alginate polymerization and modification are linked in *Pseudomonas aeruginosa*. M Bio 2015;6:e00453-15.
11. Heidari R, Farajzadeh Sheikh A, Hashemzadeh M, Farshadzadeh Z, Salmanzadeh S, Saki M. Antibiotic resistance, biofilm production ability and genetic diversity of carbapenem-resistant *Pseudomonas aeruginosa* strains isolated from nosocomial infections in southwestern Iran. Mol Biol Rep 2022;49:3811-22.
12. Jacobs HM, O'Neal L, Lopatto E, Wozniak DJ, Bjarnsholt T, Parsek MR. Mucoid *pseudomonas aeruginosa* can produce calcium-gelled biofilms independent of the matrix components Psl and CdrA. J Bacteriol 2022;204:e0056821.
13. Sato Y, Unno Y, Ubagai T, Ono Y. Sub-minimum inhibitory concentrations of colistin and polymyxin B promote Acinetobacter baumannii biofilm formation. PloS One 2018;13:e0194556.
14. Shen L, Shi Y, Zhang D, Wei J, Surette MG, Duan K. Modulation of secreted virulence factor genes by subinhibitory concentrations of antibiotics in *Pseudomonas aeruginosa*. J Microbiol 2008;46:441-7.
15. Sanz-García F, Hernando-Amado S, Martínez JL. Evolution under low antibiotic concentrations: A risk for the selection of *Pseudomonas aeruginosa* multidrug-resistant mutants in nature. Environ Microbiol 2022;24:1279-93.
16. Bruchmann J, Kirchen S, Schwartz T. Sub-inhibitory concentrations of antibiotics and wastewater influencing biofilm formation and gene expression of multi-resistant *Pseudomonas aeruginosa* wastewater isolates. Environ Sci Pollut Res Int 2013;20:3539-43.
17. Clinical and laboratory standard institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 27th ed (M100-S30). Wayne, PA: CLSI; 2020.
18. Davarzani F, Saidi N, Besharati S, Saderi H, Rasooli I, Owlia P. Evaluation of antibiotic resistance pattern, alginate and biofilm production in clinical isolates of *Pseudomonas aeruginosa*. Iran J Public Health 2021;50:341-9.
19. Ghadaksaz A, Imani Fooladi AA, Mahmoodzadeh Hosseini H, Amin M. The prevalence of some Pseudomonas virulence genes related to biofilm formation and alginate production among clinical isolates. J Appl Biomed 2015;13:61-8.
20. Powell LC, Pritchard MF, Ferguson EL, Powell KA, Patel SU, Rye PD, *et al*. Targeted disruption of the extracellular polymeric network of *Pseudomonas aeruginosa* biofilms by alginate oligosaccharides. NPJ Biofilms Microbiomes 2018;4:13.
21. Delgado C, Florez L, Lollett I, Lopez C, Kangeyan S, Kumari H, *et al*. *Pseudomonas aeruginosa* regulated intramembrane proteolysis: Protease MucP can overcome mutations in the AlgO periplasmic protease to restore alginate production in nonmucoid revertants. J Bacteriol 2018;200:e00215-18.
22. Pournajaf A, Razavi S, Irajian G, Ardebili A, Erfani Y, Solgi S, *et al*. Integron types, antimicrobial resistance genes, virulence gene profile, alginate production and biofilm formation in Iranian cystic fibrosis *Pseudomonas aeruginosa* isolates. Infez Med 2018;26:226-36.
23. Khan F, Lee JW, Javaid A, Park SK, Kim YM. Inhibition of biofilm and virulence properties of *Pseudomonas aeruginosa* by sub-inhibitory concentrations of aminoglycosides. Microb Pathog 2020;146:104249.
24. Srivastava P, Sivashanmugam K. Efficacy of sub-MIC level of meropenem and ciprofloxacin against extensive drug-resistant (XDR) *Pseudomonas aeruginosa* isolates of diabetic foot ulcer patients. Infect Genet Evol 2021;92:104824.
25. Hagras SAA, Hosny AEMS, Helmy OM, Salem-Bekhit MM, Shakeel F, Farrag HA. Effect of sub-inhibitory concentrations of cefepime on biofilm formation by *Pseudomonas aeruginosa*. Can J Microbiol 2021;67:894-901.
26. Linares JF, Gustafsson I, Baquero F, Martinez JL. Antibiotics as intermicrobial signaling agents instead of weapons. PNAS 2006;103:19484-9.
27. Navidifar T, Amin M, Rashno M. 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*. Infect Drug Resist 2019;12:1099-111.
28. Yousefpour Z, Davarzani F, Owlia P. Evaluating of the effects of sub-MIC concentrations of gentamicin on biofilm formation in clinical isolates of *Pseudomonas aeruginosa*. Iran J Pathol 2021;16:403-10.
29. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, *et al*. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and β-lactamase and alginate production. Antimicrob Agents Chemother 2004;48:1175-87.
30. Roudashti S, Zeighami H, Mirshahabi H, Bahari S, Soltani A, Haghi F. Synergistic activity of sub-inhibitory concentrations of curcumin with ceftazidime and ciprofloxacin against *Pseudomonas aeruginosa* quorum sensing related genes and virulence traits. World J Microbiol Biotechnol 2017;33:50.