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Examination of the Production of Biofilm and Acyl Homoserine Lactone (AHL) in *Pseudomonas aeruginosa* Isolates Exposed to Sub-inhibitory Antibiotics Concentrations

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Authors' contributions

This work was carried out in collaboration between all authors. Authors CK, RG and CB designed the study. Authors CK and CB performed the analysis of the study and wrote the protocol. Authors OI, CK and RG wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

It is possible to be exposed to sub-inhibitory antibiotics concentrations due to either patients or physicians during antibiotic therapy. In this study, we aimed to examine the production of biofilm and acyl homoserine lactone (AHL) in *Pseudomonas aeruginosa* isolates which were exposed to sub-inhibitory antibiotics concentrations.
In this study, The bacteria incubated in Muller Hinton agar in the oven and placed antibiotic discs onto them. As the distance increases from the antibiotic discs, the effective concentration of antibiotic decreases. Furthermore, the zone without bacterial growth ends from the place where the

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effect of antibiotic decreases to sub-inhibitory concentration and bacterial growth starts.A model by accepting this zone in which the bacteria are exposed to the sub-inhibitory antibiotic concentration have established. The four different Pseudomonas aeruginosa isolates were exposed to sub-inhibitory concentrations of amikacin, gentamicin, imipenem, meropenem, ciprofloxacin, cefepime, ceftazidime and colistin antibiotics for 5 days. Biofilm productions of isolates pre and post exposures the antibiotic were determined using microtitration plaque. Furthermore, the cross-feeding assay was performed for AHL detection.

Biofilm production was observed in all isolates exposed to 3 isolates ceftazidime when exposed to cefepime, in 3 isolates when exposed to ciprofloxacin, in 2 isolates when exposed to colistin, in 2 isolates when exposed to amikacin, in 2 isolates when exposed to gentamicin, in 1 isolate when exposed to imipenem, and in 1 isolate when exposed to meropenem. Some of these antibiotics affected the AHL production, whereas some others deteriorated the AHL production. It was observed that all of the sub-inhibitory antibiotic concentrations led to biofilm production. Some of them increased the biofilm production in all 4 isolates, whereas some others increased the biofilm production in at least 1 isolate. The most effective group in biofilm production was the cephalosporin group and less biofilm production was observed with the carbapenems (imipenem, meropenem).

We detected that biofilm production continued in strains in which there was no AHL production or the AHL production was deteriorated. This study found, that there was no relationship between the AHL production of strains and the biofilm production.

Keywords: Acyl-homoserine lactone; biofilm production; Pseudomonas aeruginosa; sub-inhibitory concentration.

1. INTRODUCTION

Pseudomonas aeruginosa is one of the most common pathogens for infections which are difficult to treat due to its increasing incidence, continuously increasing antibiotic resistance and variety of virulence factors. Furthermore, it leads to high mortality and morbidity [1,2]. Pathogenic bacteria are sometimes exposed to sub-inhibitory antibiotic concentrations (non-lethal concentrations) for a long time. This can happen due to the use of insufficient concentrations of antibiotics or inadequate concentrations of antibiotics reaching the place where the bacteria are. Bacteria found in the normal flora can be exposed to inhibitory or sub-inhibitory antibiotic concentrations during the systemic antibiotic use.

Bacteria have a sensitive perception system, “quorum sensing, (QS)” which facilitates the bacteria’s secretion of virulence factor that can ensure the communication between bacteria after meeting with the host. This system works with signal molecules depending on the cellular intensity and one of the most important signal molecules in gram-negative bacteria is N-acyl homoserine lactone (AHL) [3,4]. Biofilm production is one of the most important virulent factors and it protects the bacteria from the immune responses of the host, antibiotics and disinfectants [5,6]. In this study, the effects of the exposure to sub-inhibitory antibiotic concentrations on in vitro AHL were aimed to examined and biofilm production of P. aeruginosa isolates.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culturing Conditions

In total, 4 strains of *P. aeruginosa* isolated from patients admitted to the Amasya University Sabuncuoğlu Şerefeddin Education and Research Hospital, Turkey during the period January 2015 to January 2016 were used. Bacterial species were identified through conventional methods and additionally by VITEK 2 (bioMérieux, France) automated system. All bacterial strains were stored at −80°C in Brain Heart Infusion broth with 15% glycerol. Strains were routinely cultured in tryptic soy broth (TSB) (Merck, Germany) or tryptic soy agar (TSA) (Merck, Germany).

2.2 Antimicrobial Susceptibility Assay

In the disc diffusion method, antibiotic effect concentration decreases with increasing distance from the antibiotic disc. Bacterial growth starts from the point where the antibiotic concentrations start to have sub-inhibitory effects. This zone was accepted as the place where the bacteria are exposed to sub-inhibitory antibiotic concentrations and we established a model. In

our study, 8 different antibiotic discs were used (oxoid, UK) including amikacin (AK) (30 µg), gentamicin (GN) (10 µg), imipenem (IMP) (10 µg), meropenem (MRP) (10 µg), ceftazidime (CAZ) (30 µg), cefepime (FEP) (30 µg), ciprofloxacin (CIP) (5 µg) and colistin (CL) (10 µg). *P. aeruginosa* strain (ATCC 27853) was used as control strain. This bacterium was sensitive to these antibiotics and different clinical *P. aeruginosa* strains. These four isolates were led to be exposed to 8 different sub-inhibitory concentrations of antibiotics for five days. After maintaining these ATCC strains in the Eosin Methylene Blue (EBM) agar growth media. 0.5 McFarland standards (using 0.9% saline buffer) of these colonies were obtained and cultivated them in Mueller-Hinton agar (Oxoid, UK, agar thickness is 3 mm) using the disc diffusion method. Upon the incubation of colonies which were cultivated on the AK disc for 24 hours at 37°C, Colonies from the zones where bacteria were exposed to sub-inhibitory antibiotic concentrations were obtained. These colonies were cultivated in Mueller-Hinton Agar and AK disc was replaced on the agar and bacteria were incubated in the incubator further incubated for 24 hours. This process was repeated five more days. In this way, these bacterial colonies to sub-inhibitory concentrations of AK for five days were exposed. The same process which was explained above for *P. aeruginosa* ATCC 27853 as a control strain was also performed for other 7 antibiotics. All of these steps were repeated for 3 clinical isolates [7].

2.3 Cross Feeding Test for the Detection of Homoserine Lactone

Luria Bertani (LB) agar was covered with 40 µl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). In order to do this, X-Gal was mixed with dimethyl sulfoxide and melted. The samples were left 1-2 hours at 37°C for drying. After the LB agar plate was dried, *Agrobacterium tumefaciens* A136 and clinical *P. aeruginosa* strains were cultivated 1 cm apart from each other using the spread-plating method. *Agrobacterium tumefaciens* A136 was used indicator organisms for AHL. Color changes were observed during the 48-72 hours incubation. Incubation of *P. aeruginosa* in the presence of the indicator strain, Agrobacterium tumefaciens A136 produces a blue colour in the bioassay medium due to expression of the lacZ reporter gene [8].

2.4 Biofilm Examination Using Microtitration Method

Fresh *P. aeruginosa* cultures which were cultivated in the blood agar were obtained, transferred to tubes containing Tryptic soy broth (TSB) and 0.25% glucose, and tubes were incubated for 24 hours at 37°C. Cultures concentration were 1/20 diluted and 150 µl of them were incubated in the flat-bottomed polystyrene microtiter plates (96 well plate) for 24 hours at 37°C. Following the incubation, the plate wells were washed three times with distilled water, and the biofilm fixed by air drying. 1% crystal violet solution was added to the wells (100 µl per each) and samples were incubated for 15 minutes at room temperature. After the incubation, wells were washed with distilled water and dried by inverting the plates onto the blotter paper. 150 µl ethanol/acetone (80:20) solution was added to the wells, incubated 10 minutes and the dye was dissolved. Absorbance values were measured at 570 nm by ELISA reader. The same isolate was divided into three different wells and absorbance values of these separate wells were also measured. The mean values were calculated. In our study, we compared the absorbance values (570 nm by ELISA reader, Thermo, Multiskan, GO) of the *Escherichia coli* (*E. coli*) ATCC 25922 strain as a negative control which did not produce biofilm and *P. aeruginosa* ATCC 15692 which produced biofilm [9].

3. RESULTS

We examined the biofilm production in 5 different *P. aeruginosa* isolates (4 clinical isolates and 1 ATCC 15692 *P. aeruginosa*). According to our results, there was biofilm production in all of the strains exposed to ceftazidime, in 3 of them exposed to cefepime, in 3 of them exposed to ciprofloxacin, in 2 of them exposed to colistin, in 2 of them exposed to amikacin, in 2 of them exposed to gentamicin, in 1 of them exposed to imipenem, and in 1 of them exposed to meropenem. There was a decreased biofilm production in one of the strains exposed to amikacin and in another strain exposed to colistin (Table 1).

Pre-exposure the antibiotic, there was an AHL production only in the third isolate, whereas there was no AHL production in the other 3 isolates. AHL production was detected after the ciprofloxacin exposure of the first isolate. Furthermore, there was an AHL production after

Table 1. Examination of biofilm productions of *P. aeruginosa* strains pre and post exposures antibiotic using microtitration method (These values are absorbance)

Strains	The pre-exposure	The Post-exposure							
		CAZ	CPM	AK	GN	IMP	MRP	CIP	CL
1	0.3037 (++)	0.7697 (+++)	0.3348 (++)	0.4575 (++)	0.2911 (++)	0.2990 (++)	0.3281 (++)	0.4304 (++)	0.0577 (-)
2	0.4364 (++)	0.5174 (+++)	0.6876 (+++)	0.1889 (+)	0.3155 (++)	0.3158 (++)	0.3367 (++)	0.6254 (+++)	0.5122 (+++)
3	0.3583 (++)	0.5298 (+++)	1.0771 (+++)	0.6025 (+++)	0.6107 (+++)	0.3840 (++)	0.9732 (+++)	0.5899 (+++)	0.6278 (+++)
4	0.1420 (-)	0.1788 (+)	0.3369 (++)	0.7760 (+++)	0.6060 (+++)	0.1813 (+)	0.1274 (-)	0.8278 (+++)	0.4536 (++)

Positive control: P. aeruginosa (ATCC 15692): 0,5622 (+++)
Negative control: E. coli (ATCC 25922): 0,1442 (-)
CAZ: Ceftazidime, CPM: Cefepime, AK: Amikacin, GN: Gentamicin, IMP: Imipenem, MRP: Meropenem, CIP: Ciprofloxacin, CL: Colistin. OD570<0.1440 (-:non-biofilm), OD570<0.288 (+: weak biofilm formation), OD570<0.500 (++: moderate biofilm formation), OD570>0.500 (+++:strong biofilm formation)

Table 2. AHL production pre and post exposure antibiotic of *P. aeruginosa* isolates

Strains	Pre-exposure	Post-exposure							
		CAZ	CPM	AK	GN	IMP	MRP	CIP	CL
1	-	-	-	-	-	-	-	+	-
2	-	-	-	-	-	-	-	-	-
3	+	+	+	-	-	+	+	+	+
4	-	-	-	-	-	-	-	-	+

CAZ: Ceftazidime, CPM: Cefepime, AK: Amikacin, GN: Gentamicin, IMP: Imipenem, MRP: Meropenem, CIP: Ciprofloxacin, CL: Colistin. -: Negative, +: Positive.

the colistin exposure of fourth strain. However, antibiotic exposure did not lead to AHL production in the second strain. It was observed that AHL production was deteriorated in the third strain due to amikacin and gentamicin treatment and thus the bacterial growth was inhibited (Table 2).

According to our results, almost all of the sub-inhibitory concentrations of antibiotics can lead to biofilm production. Some of them increased the biofilm production in 4 of the strains and some others increased the biofilm production in at least one strain. The most effective group on biofilm production was the cephalosporin group and less biofilm production was observed with the carbapenems (imipenem, meropenem) (Table 1). It was observed that there was no difference between the biofilm and AHL productions of control ATCC strains which were cultivated for five days.

4. DISCUSSION

Antibiotics are one of the most important discoveries in the history of humanity and they have been successfully used in the treatment of various infectious diseases. However, inappropriate and unnecessary use of antibiotics significantly diminishes their effects due to antibiotic resistance. Microorganisms sooner or later develop resistance against agents which are used to kill them. This resistance against antimicrobial agents is a very important issue which can threaten all humanity. A resistance can be developed in any microorganism which already developed resistance against another antimicrobial agent with similar effect mechanisms [10]. Pathogenic bacteria are sometimes exposed to sub-inhibitory antibiotic concentrations (non-lethal concentrations) for a long time. This can happen due to the use of insufficient concentrations of antibiotics or inadequate concentrations of antibiotics reaching the place where the bacteria are. Furthermore, bacteria present in the normal body flora can be exposed to sub-inhibitory concentration antibiotics. *P. aeruginosa* is commonly observed either as pathogenic or as a member of the human flora and it is one of the bacteria which can be frequently exposed to sub-inhibitory concentrations of antibiotics. QS system determines the intensity of bacteria via cellular communication signals among cells upon

meeting the host and this system ensures bacteria change their responses via signals according to their intensities [11]. AHL production is one of the most common communication systems as depending QS system. As other Gram negative bacteria, an important part of the *P. aeruginosa* isolates use these diffused signal molecules [12,13]. QS system regulates the production of various virulent factors such as elastase, protease, exotoxin A pyocyanin, cyanide and alkaline protease in addition to biofilm production [14]. The environmental detection system of *P. aeruginosa* has roles not only in the production of virulent factors but also in the realization of the swimming, sliding, and vibration motions [15]. It is obvious that the QS system causes a prominent decrease in bacterial virulence. Mutant strains with a deteriorated QS system cannot colonize the cornea in the mouse traumatized eye model and cannot lead to corneal damage [16]. The AHL production of *P. aeruginosa* isolates was examined post-exposure the sub-inhibitory antibiotic and the AHL production was observed in the first isolate treated with ciprofloxacin. AHL production was detected in the fourth isolate with the effect of colistin. On the other hand, antibiotic exposure did not lead to AHL production in the second isolate. Amikacin and gentamicin exposure deteriorated and inhibited the AHL production in the third isolate. It is interesting that these two aminoglycoside type antibiotics inhibit the AHL production in bacteria (Table 2). Biofilm production is one of the most important virulent factors of *P. aeruginosa* and it is controlled by the QS system. Biofilm protects the bacteria from the phagocytosis and effects of complements. Bacteria are resistant against antibiotics and disinfectants inside the biofilm layer. Therefore, biofilm production plays a critical role in the continuity of the bacteria in the host and the external environment [17,18]. Sticky bacterial cells have antigenic features and they increase the antibody production. Antibodies cannot kill bacteria surrounded by biofilm and they also lead to immune complex damage in neighboring tissues [19,20]. Biofilms are also risk factors for surgical prostheses; biofilm layers damage the surface of the prosthesis and allow infections. In this way, biofilm layers shorten the life of the prosthesis, and increase the treatment costs [7,21,22].

It has recently been shown that the expression of secretion efflux pumps of various microorganisms, including *P. aeruginosa,* increases in the presence of biofilm [23]. It is very hard to treat biofilm producing *P. aeruginosa* strains. Therefore, infections of *P. aeruginosa* lead to high mortality rates and the treatment costs are very high. Previous studies have, indicated that sub-inhibitory concentrations of aminoglycosides and imipenem in *P. aeruginosa* isolates lead to increases in exopolysaccharide alginate production and biofilm production [24,25].

Biofilm production was examined in 4 different *P. aeruginosa* isolates (4 clinical strains and 1 standard strain). According to our results, there was biofilm production in all of the strains exposed to ceftazidime, in 3 of them exposed to cefepime, in 3 of them exposed to ciprofloxacin, in 2 of them exposed to colistin, in 2 of them exposed to amikacin, in 2 of them exposed to gentamicin, in 1 of them exposed to imipenem, and in 1 of them exposed to meropenem (Table 1).

According to our results, all of the sub-inhibitory concentrations of antibiotics can increase biofilm production. Some of them increased biofilm production in all of the 4 strains and others increased biofilm production in at least one of the isolates. Cephalosporins (ceftazidime, cefepime) were shown to be effective in biofilm production. Moreover, biofilm production was less affected by carbapenem type antibiotics (imipenem, meropenem) (Table 1).

In this study, we detected that exposure to sub-inhibitory antibiotic concentrations has effects on AHL production. According to our findings, some antibiotics triggered AHL production, whereas others deteriorated AHL production. This made us think that there may be a different signal molecule besides AHL in some of the isolates. We detected that biofilm production continued in strains in which AHL was not produced or AHL production was deteriorated. We did not detect any relationship between AHL and biofilm production in our strains.

Our options to combat bacterial infections with antibiotics are gradually being depleted. Therefore, we can combat bacterial infections by preventing AHL production, which is an important signal molecule of the QS system and which is commonly used by bacteria in order to maintain their vitality and secrete virulent factors. In our study, it is shown that antibiotic exposures can either induce or inhibit AHL production of bacteria. According to our findings, the underlying mechanisms can be comprehensively

clarified using molecular methods and with higher numbers of isolates.

5. CONCLUSION

Antibiotics do not only kill bacteria; they also have other effects on them. The use of antibiotics with sub-inhibitory concentrations can trigger the strains to produce more biofilm during the treatment and creates problems in the infection therapy. Furthermore, sub-inhibitory antibiotic concentrations can induce the normal flora bacteria to produce higher amounts of biofilm and they can become serious potential for future infections.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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