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Research article

Variations in *Pseudomonas aeruginosa* Biofilm Formation Influence Virulence and amoxicillin Resistance

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ABSTRACT

Inhibition of adherence pathogenic bacteria, such as Pseudomonas aeruginosa, to the surfaces is one important approach that is employed by antibiotics against bacterial infectious diseases. The present study is aimed at determining the involvement of sub-inhibition concentrations of amoxicillin (AMX) in the modulation of P. aeruginosa capacity to produce the biofilm. The approach was to expose the P. aeruginosa to various sub-minimum inhibitory concentrations (MICs) of amoxicillin (AMX) and thereafter test the resultant biofilm formation by the bacteria under these exposure conditions. The research also determined the effect that sub-MICs of AMX have on surface hydrophobicity and bacterial aggregation. The findings were that sub-MICs of AMX influenced varying forms of biofilm formation. High sub-MIC of AMX (½ MIC) lowered the formation of the biofilm, while low sub-MICs enhanced the capacity for the bacteria to develop polystyrene microtiter plate biofilms. The influence that sub-MICs of AMX have on surface hydrophobicity and bacterial aggregation detection were similar to what they showed on the production of the biofilm. Low level sub-MICs concentrations of AMX ($^1/_{16}$, $^1/_{32}$, and $^1/_{64}$) enhanced surface hydrophobicity, while on the other hand ½ MIC AMX lowered the surface hydrophobicity of the bacteria. Sub-MICs of AMX ($^1/_{8,}$ $^1/_{16}$, $^1/_{32}$, and $^1/_{64}$) also enhanced the rate of bacterial aggregation for P. aeruginosa. The study concludes that sub-MICs of AMX improve the capacity for P. aeruginosa to produce biofilms by enhancing surface hydrophobicity and bacterial aggregation.

Keywords: Aggregation, Amoxicillin, Biofilm, Hydrophobicity, Pseudomonas aeruginosa.

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1. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that has high clinical importance because of its ability to cause different infections in immunocompromised patients and those with basic conditions, including cystic fibrosis, wounds, and burns [1]. It was found that adaptability and intrinsic resistance to several classes of antimicrobial agents make it a major reason of acquired infections, such as ventilator-associated lung infections, bacteremia, and urinary tract infections (UTIs) that link with catheter [2]. The biofilm formation by P. aeruginosa is a vital factor contributing to its persistence and resistance to different types of antibiotics [3]. *Biofilms* structurally form microbial communities implanted in an exopolysaccharide biomass that forms the metabolic activity of bacteria that attach to the different types of the surfaces [4]. In the pathogenic bacterium, the biofilm polymer matrix improves bacterial survival by restricting antibiotic penetration, facilitating gene transfer, and shielding cells from host innate and acquired immune responses [5]. This shielding environment enhances the development of persistent infections, making treatment problematic and leading to unsuccessful treatment outcomes [6]. Furthermore, the difference between the

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biofilm-forming cells and planktonic cells is the level of resistance to antibiotics and the modulation of virulence factor expression [7]. Variation in the *P. aeruginosa* strains to produce biofilms is increasingly recognized as a key factor in clinical outcomes. Strains that produce more biofilm are often associated with more persistent infections, higher resistance levels, and increased virulence [8.9]. Conversely, strains with weaker biofilm formation tend to be less pathogenic but can still be clinically relevant due to other resistance mechanisms [10]. Understanding how differences in biofilm formation impact virulence and antibiotic resistance is essential for unraveling the pathogenic success of *P. aeruginosa* [11].

This *study* aims to explore the differences in biofilm formation among *P. aeruginosa* strains and their relationship with virulence and antibiotic resistance by analyzing the interaction between biofilm production, pathogenicity, and antimicrobial susceptibility.

2. MATERIALS and METHODS

2.1. Bacterial Strains and Activation

The isolate of *P. aeruginosa* procured from the microbiological Lab at the Department of Biology, University of Baghdad. The isolate was routinely subcultured on nutrient agar every week for short-term preservation. The bacterial isolate of *P. aeruginosa* was stored for the long term in nutrient broth (supported with 20% glycerol) at -20 ºC.

2.2 Minimum Inhibitory Concentration

The method that described by Al-Mutalib and Zgair (2023) was followed to measure the minimum inhibitory concentrations (MICs) of amoxicillin (AMX, AdvaCare Pharma, USA) against *P. aeruginosa*. The MIC was defined as the lowest concentration of the amoxicillin (AMX) that totally inhibited visible growth of *P. aeruginosa* [12].

2.2 Biofilm Formation

The *P. aeruginosa* in Tryptic soy broth (Himedia, TSB) for 18 h was washed, and the density of bacterial suspension was adjusted with TSB to be 0.1 at 600 nm. Hundred microliters of TSB were added to each well of a flat shape polystyrene microtiter plate (Nunc, Denmark), and then 10 µL of bacterial inoculum was applied to the wells. The plates were incubated for 18 h at 37 ºC. After incubation, the plates were washed with distilled water. The plates were dried at 55 ºC for an hour, and then 100 µL of crystal violet (Himedia) was added to each well. The plates were washed three times with distilled water after incubation at 21 ºC for 15 min. One hundred microliters of 99% ethanol (Fluka) were added to each well. The absorptance was taken at 590 nm using a microplate reader (BioTek 800, USA). TSB medium without any bacterial solution was used as a blank control [13]. Similar method was repeated using serial dilution of Sub-MICs of amoxicillin.

2.3. Surface Hydrophobicity of Bacteria

The microbial hydrocarbon adsorption capacity technique was used to measure the hydrophobicity of bacterial surfaces [14]. This method was done to the bacterial suspension exposed to the antibiotic (amoxicillin) and the results was compared with bacterial suspension exposed to PBS (pH 7.2; 0.1 M) The overnight bacterial culture of *P. aeruginosa* in TSB (Himedia) was centrifuged at 9000 g (Eppendorf, Germany) for 7 min, and then washed three times with phosphate-buffered saline (PBS, pH 7.2; 0.1 M). The absorptance of the bacterial suspension was changed to 0.9 at OD600 nm (OD1). Bacterial suspension and hydrophobic solvent (chloroform/xylene, Sigma-Aldrich) were mixed in a ratio of 5:1 in tubes and left at 26 ◦C for 90 min, with PBS (blank control), and each sample was repeated 3 times, and the OD600 nm was measured (OD2). The surface hydrophobicity was calculated using the following equation:

$$Surface\ hydrophobicity = (1 - \frac{OD2}{OD1})\ x100\%$$

2.4. Self-Agglutination Rate

The bacterial suspension was prepared according to the previous step of 2.3. The optical density of the bacterial inoculum was recorded as OD1. Another tube, composed of 5 mL of the same bacterial suspension with the same optical density and optical density at 600 nm, was prepared from the bacterial suspension after exposure to sub-MICs of amoxicillin, and the optical density was recorded as OD2. Dependent on the difference in absorbance of the bacterial solution before and after standing, the self-agglutination rate of conditioned *P. aeruginosa* isolate (exposed to antibiotics) was calculated [15]:

$$Self - agglimerate = (1 - \frac{OD2}{OD1})\ x100\%$$

2.7. Statistical Analysis

The data was conducted using Student's t-test and one-way analysis of variance (ANOVA). The *OriginLab® Release of Origin® 8.6 was used to analyze the data*. $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1. Effect of AMX on Biofilm Formation

In this experiment the MICs of amoxicillin against *P. aeruginosa* was measured. The MIC of amoxicillin was 500 µg/ml. The effect of different sub-MIC concentrations of amoxicillin on the biofilm production of *P. aeruginosa* was evaluated in the current study. It was found that the different sub-MICs of amoxicillin had an effect on the biofilm formation of *P. aeruginosa* in different ways according to the concentrations that were used.

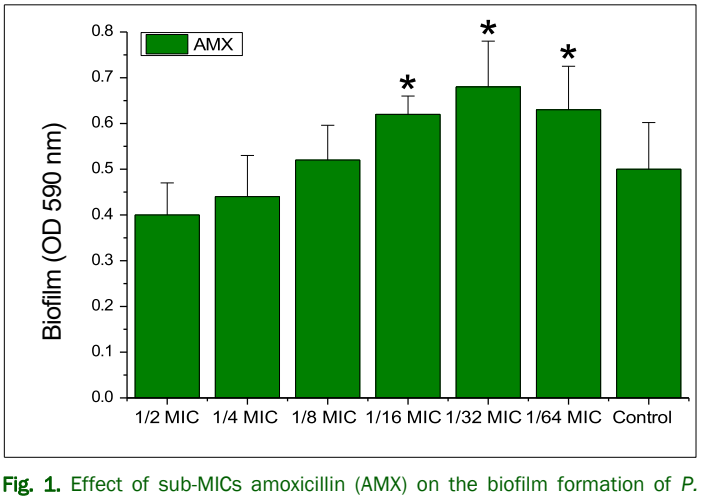

Fig. 1. Effect of sub-MICs amoxicillin (AMX) on the biofilm formation of *P. aeruginosa* on polystyrene microtiter plates. Asterisks indicate a significant difference from the control group (*P. aeruginosa* treated with PBS, without antibiotic stress).

The highest MIC concentrations of amoxicillin (½ and ¼ MICs) reduced biofilm formation, but the reduction was not significant compared with the control (biofilm formation of *P. aeruginosa* without antibiotic stress). The lowest concentrations of sub-MICs

AMX (1/16, 1/32, and 1/64) enhanced the biofilm formation significantly compared with the control.

3.2. Surface Hydrophobicity

Fig.2. depict the rate of hydrophobicity of *P. aeruginosa* treated with different concentrations of amoxicillin, the results were compared with the rate of hydrophobicity of *P. aeruginosa* treated with PBS (no antibiotic stress). The results showed a significant decrease in the hydrophobicity rate in *P. aeruginosa* treated with ½ MIC amoxicillin, while no effect of ¼ and 1/8 MIC amoxicillin on the *P. aeruginosa* hydrophobicity rate. The significant elevation of the hydrophobicity rate was seen when the *P. aeruginosa* was treated with 1/16, 1/32, and 1/64 MIC amoxicillin ($P<0.05$).

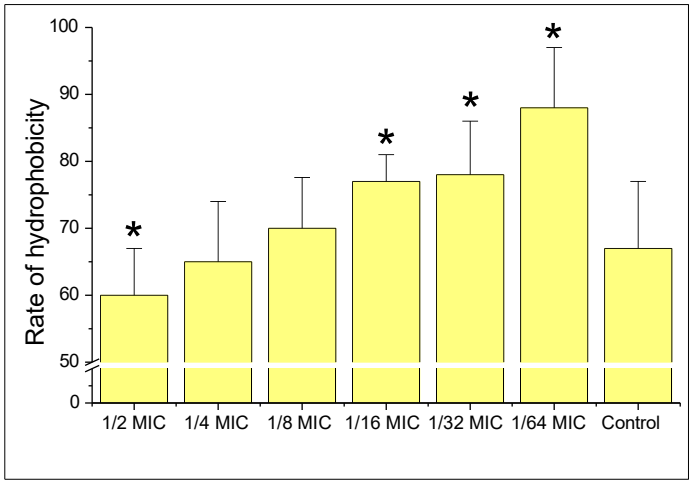

Fig.2. Detection of surface hydrophobicity of *P. aeruginosa* exposed to different sub-MICs of amoxicillin. The rate of hydrophobicity of *P. aeruginosa* exposed to PBS represents the control. Asterisks indicate a significant difference from the control group

3.3. Rate of Self-Aggregation

The result of the present study showed the self-aggregation capacity of bacterial cells of *P. aeruginosa* treated with different sub-MICs of amoxicillin. The results showed that a significant elevation of aggregation rate was seen in the *P. aeruginosa* that was treated with 1/8, 1/16, 1/32, and 1/64 MICs of amoxicillin, $P<0.05$. No effect of the highest concentrations of MICs of amoxicillin on the aggregation capacity of *P. aeruginosa*.

4. DISCUSSION

The current study emphasizes the complex and concentration-dependent effects of sub-inhibitory levels of amoxicillin (AMX) on *P. aeruginosa's* ability to form biofilms. Conventional antibiotic treatments aim to reduce bacterial growth; however, exposing bacteria to sub-MICs often produces conflicting results that may enhance pathogenicity rather than suppress it [12, 17,18]. Our findings showed that low sub-MIC levels of AMX significantly promoted biofilm formation, surface hydrophobicity, and bacterial aggregation, while higher sub-MIC levels (½ MIC) inhibited these processes.

These results align with previous reports that sub-MICs of β-lactam antibiotics can alter the physiology and virulence factor production in *P. aeruginosa* [19]. Low sub-MICs of AMX seem to promote initial adhesion and biofilm maturation by increasing cell surface hydrophobicity, which enhances stronger surface interactions with abiotic surfaces like polystyrene. This induction of adhesion correlates with increased bacterial aggregation, potentially contributing to microcolony formation and stabilization of the biofilm matrix [20]. The reduction in hydrophobicity and aggregation at higher sub-MIC levels is consistent with decreased biofilm formation, indicating that near-inhibitory sub-MICs of AMX may disrupt surface interactions necessary for effective colonization.

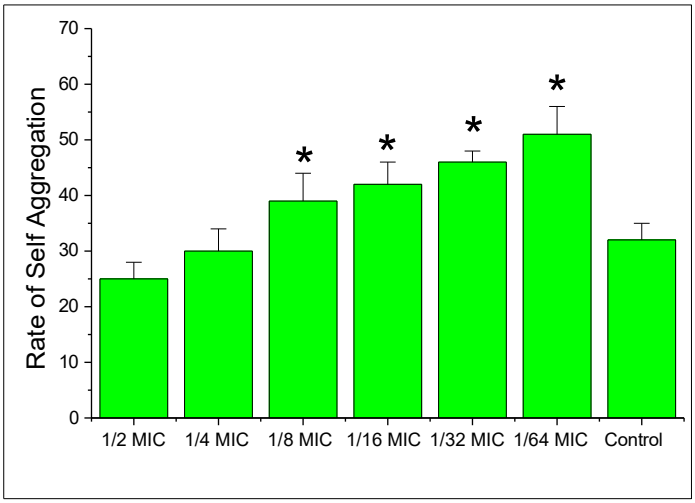

Fig.3. Detection of the aggregation rate of *P. aeruginosa* treated with different concentrations of MICs of amoxicillin. The control is the aggregation rate of *P. aeruginosa* treated with PBS. Asterisks indicate a significant difference from the control group

The concentration-dependent dual effect of AMX highlights the delicate balance between bacterial adaptation and antibiotic pressure. At sub-MIC levels of lower magnitude, bacteria perceive AMX as stress and activate adaptive regulation, leading to biofilm-based survival strategies. The formation of biofilm in this context is an adaptive response that makes bacteria more tolerant of antibiotics and host defenses. At higher sub-MIC levels, these adaptive responses may be weakened, resulting in decreased adherence and compromised biofilm stability [21].

Clinically, these observations are particularly concerning. Biofilms formed under low-dose antibiotic enhance the resistant to treatment that used the antibiotics and also resistance the host immune response. This may lead to chronic infections, especially in immunocompromised patients or those with indwelling medical devices. Additionally, biofilm-associated cells display phenotypic resistance, which can decrease the effectiveness of subsequent antibiotic treatments [22]. Therefore, using inappropriate antibiotic dosages may unintentionally promote persistence and complicate infection management.

The results emphasize the link between hydrophobicity, cell aggregation, and biofilm formation. The observed increase in surface hydrophobicity and cell aggregation at sub-MIC levels of the chemical suggests that these phenotypic traits are key in initiating biofilm development. This aligns with previous findings indicating that hydrophobic bonding facilitates bacterial attachment to abiotic surfaces, while aggregation enhances biofilm structure by forming compact microcolonies [23]. The decline in these two traits at higher chemical concentrations likely explains the impaired biofilm formation seen under these conditions.

Taken together, this study demonstrates the vitality of examining the unexpected effects of antibiotics at sub-inhibitory exposure levels. Although AMX effectively inhibits the growth of *P. aeruginosa* at therapeutic doses, improper dosage or suboptimal PK (Pharmacokinetics) could increase virulence through enhanced biofilm formation [24]. Further research is needed to

investigate the molecular processes activated in response to sub-MICs of AMX, including the regulation of quorum sensing and cell surface modifications. Such data may provide the foundation for developing countermeasures against biofilm-related tolerance and preventing therapy failure [20].

5. CONCLUSION

In conclusion, this research demonstrates that sub-MICs of AMX produce dual effects on *P. aeruginosa* biofilm formation through changes in surface hydrophobicity and cell aggregation. The highest concentration of AMX reduced the biofilm formation, while the lowest sub-MIC concentrations enhanced the biofilm formation. Similar finding was seen when the surface hydrophobicity and aggregation of *P. aeruginosa* were measured when exposed to different sub-MIC concentrations of AMX. These findings not only clarify the mechanism of biofilm induction by antibiotics but also emphasize the risk of sub-therapeutic antibiotic levels in the clinical environment.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

The Ethics Committee of the University of Baghdad approved this study (CSEC/1124/0334a; November 20, 2024). Since it was a retrospective analysis of routinely collected clinical data, individual patient consent was obtained before sample collection, in accordance with national ethical guidelines.

CRediT authorship contribution statement

Saleh FM: Investigation, Methodology, Project administration, Resources, Roles/Writing – original draft, Supervision, Validation, Writing–review & editing
Mohammed HA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing
All authors have read and agreed to the published version.

Availability of data and materials

Data will be made available on request

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