Doi.org/10.36721/PJPS.2024.37.6.REG.1609-1614.1

Relationship between biofilm formation of *Pseudomonas aeruginosa* and susceptibility to rifaximin and ofloxacin

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Abstract: *Pseudomonas aeruginosa is* responsible for many infectious diseases related to antibiotic resistance. Biofilm formation may help bacteria to pass the treatment with antibiotics and overcome the immune system. Here, the relationship between antibiotics (rifaximin and ofloxacin) susceptibility and biofilm formation was evaluated. Ten isolates of *P. aeruginosa* were isolated from 110 urine specimens obtained from urinary tract infections (UTIs) patients. The spectrophotometric method was used to measure biofilm formation on polystyrene microtiter plates. Inhibitory zone onto agar plates was used to measure the antibiotic susceptibility of rifaximin and ofloxacin against the ten *P. aeruginosa* (Pa1, Pa2, Pa3, Pa15, Pa18, Pa22, Pa23, Pa25, Pa26 and Pa27) isolates. The highest inhibition zones were seen against Pa 25 and Pa 3 respectively, while the lowest inhibition zones of rifaximin were seen against Pa3, Pa23 and Pa25. Moreover, the lowest inhibition zones of both antibiotics were seen in the cases of Pa1 and Pa27 respectively. The maximum biofilm formation was seen in the case of Pa3, while Pa27 produced the lowest biofilm. The study showed no relationship between biofilm formation and susceptibility to rifaximin and ofloxacin (P>0.05).

Keywords: Biofilm formation, *Pseudomonas aeruginosa*, Rifaximin, ofloxacin.

Submitted on 08-11-2023 – Revised on 13-03-2024 – Accepted on 20-06-2024

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is present in different ecosystems and it may infect people and cause different infectious diseases. The production of biofilm by *P. aeruginosa* on both biotic and abiotic surfaces is one of its unique features. A complex population of bacterial cells in a particular space is attributed to the accumulation of the extracellular polymeric protective matrix. Biofilms are connected to persistent illnesses that are frequently antibiotic-resistant and challenging to get rid of the bacteria that formed biofilm. The biofilms contribute to the development of infections that result in major consequences and make it challenging to treat the infection with bacterial species (Lee and Yoon, 2017; de Sousa *et al*., 2021; Kassa and Al-Sayidi, 2023).

It can be challenging to treat *P. aeruginosa* infections by using antibiotics, removing infected equipment and surgical debridement. The most crucial method for treating bacterial infections is the use of antibiotics. Recently, the main issue in treating bacterial infectious diseases is rising in antibiotic resistance. Antibiotics are used not only to kill bacteria, but also studies have shown that using sub-inhibitory concentrations of antibiotics helps to reduce bacterial adhesion to different surfaces, and this will help significantly to decrease the virulence of bacteria (Tagliaferri *et al*., 2019; Ghafil *et al*., 2022).

Broad-spectrum antibiotics (rifaximin and ofloxacin) are used to treat hepatic encephalopathy, traveler's diarrhea, and other infectious diseases (Koo and DuPont, 2010; Axtell *et al*., 2018). Urinary tract infections and other bacterial infections can be effectively treated with ofloxacin, a quinolone antibiotic (Koo and DuPont, 2010). A previous study investigated the role of rifaximin in decreasing *Klebsiella pneumoniae* colonization in the gut of the experimental animal (Xenofontos *et al*., 2022). In earlier research, neither antibiotic has been shown to have any effect on biofilm formation by *P. aeruginosa*. Therefore, our current study highlighted the effect of sub-inhibitor concentrations of rifaximin and ofloxacin (0.5xMIC, 0.25xMIC, 0.125xMIC and 0.06xMIC) on the biofilm formation by *P. aeruginosa* on polystyrene.

MATERIALS AND METHODS

P. aeruginosa

Ten-milliliter urine samples were aseptically obtained from 110 UTI patients at the Baghdad Teaching Hospital, Baghdad, Iraq. A 1.0ml urine sample was incubated for two days at 37ºC with shaking at 220 rpm in 9 ml of Pseudomonas asparagine broth medium (Himedia, India). Then, 0.1ml of the prior culture was applied to the cetrimide agar (1.5% agar, Himedia, India) and incubated at 37ºC until colonies developed (Ghafil and Zgair, 2022). VITEK 2 Densi Check instrument, fluorescence system (bioMe´rieux) (ID-GNB card) was used to identify the *P. aeruginosa* isolates (Ali and Zgair, 2022). The bacterial subculture were made every week (Zgair, 2012a).

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Antibiotics susceptibility test

The antibacterial effect of rifaximin and ofloxacin against 10 isolates of *P. aeruginosa* was evaluated using agar well diffusion (Pa1 to Pa27). The standard inoculums of ten isolates of *P. aeruginosa* ($O.D.^{600\ nm}$: 0.1) were spread into the surface of sterile Mueller Hinton agar plates. Eight wells [8 millimeters (mm) in diameter] by Cork Borers were done. The wells were filled with 50 µl of 0.5 mg/ml and 1 mg/ml of the antibiotics and then the plates were incubated at 37°C for overnight. The diameter of the inhibitory zone was measured in mm (Ahmed *et al*., 2023). The method was correlated with the standards of CLSI (CLSI, 2020).

Biofilm

The flat-bottom wells of polystyrene plates were filled with 200 microliters of sterile Tryptic soy broth (TSB). Standard inoculum of 5µl of different *P. aeruginosa* (ten isolates) ($OD^{600\ nm}$, 0.1) was added to the wells and incubated at 37°C for 18 h. Control wells were filled with only 200 µl of sterile TSB. After discarding the media, non-attached bacterial cells were removed by giving them four thorough washes in distilled water. The spectrophotometric technique was used to quantify the amount of biofilm at the optical density (OD) of 570 nm. The filled wells with only TSB were considered a control (Zgair and Chhibber, 2011; Zgair and Chhibber, 2013; Ghafil, 2018).

Ethical approval

This research was approved by the University of Baghdad, Baghdad, Iraq (No 223, 2023). Every person has granted consent to participate in the research project.

STATISTICAL ANALYSIS

Origin 8 software was used for graphical representation and statistical analysis (Zgair, 2012b). The data was presented as means ± SE. The Pearson correlation coefficient was used to identify the relationship. $P<0.05$ was considered as statistically significant.

RESULTS

P. aeruginosa isolates

Ten isolates of *P. aeruginosa* were isolated and used in the further experiments of the current study.

Rifaximin susceptibility test

In the current study, the antibacterial effect of two concentrations of rifaximin against ten isolates of *P. aeruginosa*. The results showed that both 0.5 and 1mg/ml concentrations of rifaximin the results were the same and the highest antibacterial effects of this antibiotic were observed in the case of Pa25 (fig. 1). While the lowest inhibitory zone was seen in the case of Pa1 isolate (Zero mm at 0.5mg/ml of rifaximin) and in the case of Pa18 isolate (9.5 mm at 1mg/ml of rifaximin).

Ofloxacin susceptibility test

In the current study, the antibacterial effect of two concentrations of ofloxacin against ten isolates of *P. aeruginosa* was checked. The highest antibacterial effect of ofloxacin was seen in the concentrations of 0.5mg/ml and 1.0mg/ml in the case of Pa 3. The lowest antibacterial effect was seen against Pa 27 isolate at 0.5 mg/ml (no inhibitory zone) and at 1mg/ml of ofloxacin (inhibitory zone was 15mm). The present study showed that the antibacterial effect of 1.0 mg/ml of ofloxacin was higher than the antibacterial effect of 0.5mg/ml (fig. 2).

The results showed that the diameter of inhibition zone around the wells filled with either 0.5mg/ml or 1 mg/ml of ofloxacin against the studied isolates (10 isolates) was significantly higher that the diameter of inhibition zone that around the wells filled with either 0.5mg/ml ($P<0.05$) or 1mg/ml ($P<0.01$) of rifaximin against the same isolates (fig. 3). This leads to the conclusion that the sensitivity of *P. aeruginosa* to ofloxacin is higher than to rifaximin.

Biofilm formation

Fig. 4 shows the biofilm formation by different isolates of *P. aeruginosa*. The results showed that the Pa3 isolate produced the maximum level of biofilm followed by Pa15, while the Pa27 produced the lowest biofilm level.

Correlation between biofilm formation and antibiotic susceptibility

In the present study, the correlation between the biofilm formation of ten isolates of *P. aeruginosa* and antibiotic susceptibility was evaluated. fig. 5 shows no significant correlation between the biofilm formation at OD^{570nm} by *P. aeruginosa* (Pa1, Pa2, Pa3, Pa15, Pa18, Pa22, Pa23, Pa25, Pa26 and Pa27) and the rifaximin susceptibility in terms of diameter of the inhibition zone around the wells filled with either 0.5mg/ml (r:-0.077; $P>0.05$) or 1.0 mg/ml of rifaximin (r:-0.059; P: >0.05). The result showed that the biofilm formation by the ten isolates of *P. aeruginosa* was not correlated with *P. aeruginosa* resistance to rifaximin that help in the conclusion that the biofilm is not mainly responsible for the bacterial resistance to rifaximin. In current study, it was observed that the positive correlation between the biofilm produced by the 10 studied isolates and ofloxacin susceptibility in terms of the diameters of inhibition zones around the wells filled with either 0.5mg/ml of ofloxacin (r: +0.52; $P>0.05$) or 1.0mg/ml of ofloxacin (r: + 0.51; $P>0.05$) but these relationships were no significant statistically ($P\geq0.05$) (fig. 6). This shows the limited role of the biofilm in the resistance of the studied to the ofloxacin.

DISCUSSION

Antibiotic resistance and biofilm formation are closely related, as biofilms can increase the ability of bacteria to resist antibiotics. Besides helping bacterial isolates to resist the different kinds of antibiotics it also involves the attachment to a surface.

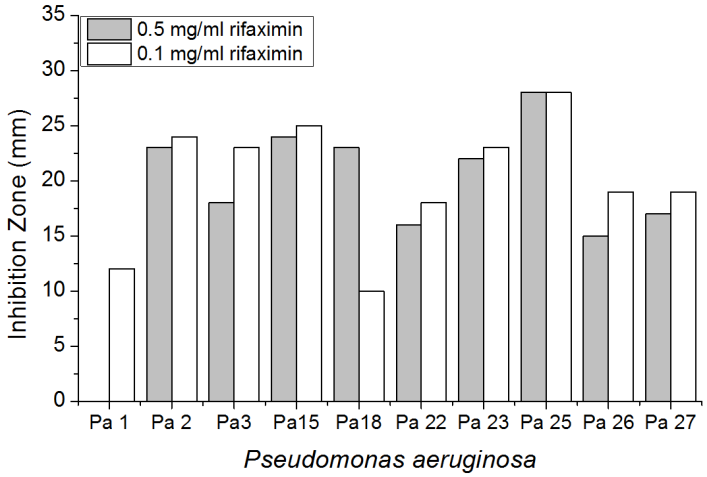

Fig. 1: The diameter of the Inhibition zone in millimeters (mm) of ten isolates of *P. aeruginosa* around the wells made in agar and filled with either 100 µl of 0.5 mg/ml or 1 mg/ml of rifaximin.

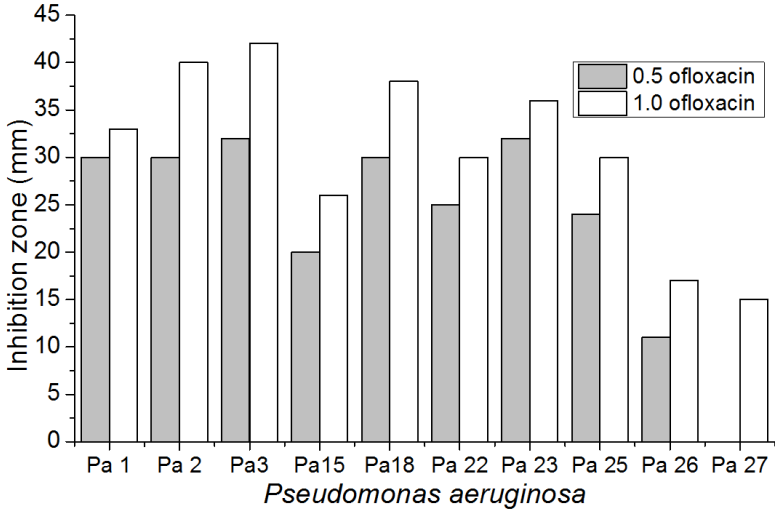

Fig. 2: Inhibition zone of ten clinical isolates of *P. aeruginosa* around the wells made in agar and filled (100 µl) of 0.5 mg/ml and 1 mg/ml of ofloxacin [The inhibition zone was measured in millimeters (mm) in diameter].

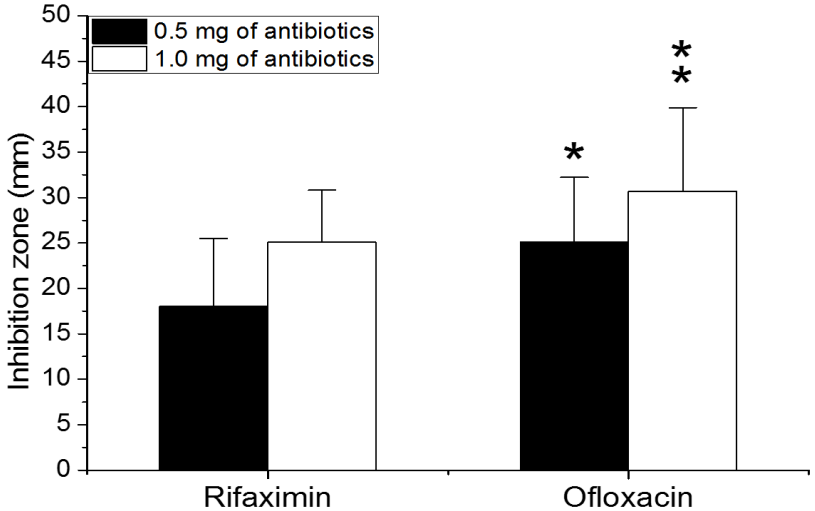

Fig. 3: The mean of diameter of inhibition zone produces by rifaximin and ofloxacin against 10 isolates of *P. aeruginosa.* The agar well diffusion method was used. The asterisks indicate the significance from the diameter of inhibition zone caused by rifaximin. *,P<0.05; **,P<0.01.

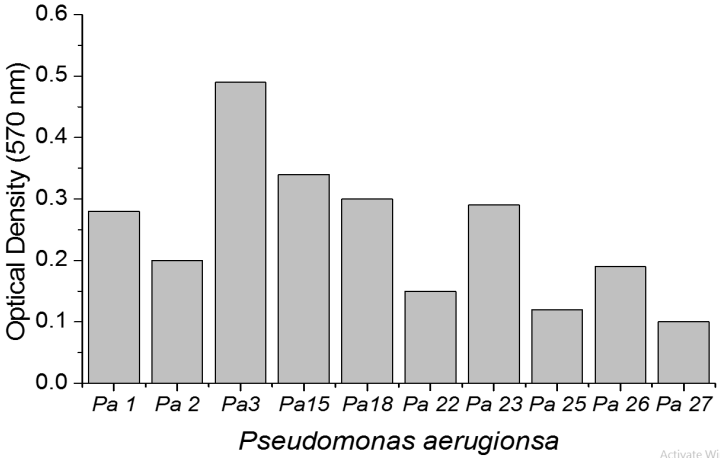

Fig. 4: Biofilm formation of ten clinical isolates of *P. aeruginosa* (Pa1, Pa2, Pa3, Pa15, Pa18, Pa 22, Pa 23, Pa 25, Pa 26 and Pa 27) to polystyrene microtiter plates that were isolated from the urine samples collected from patients suffering from UTI.

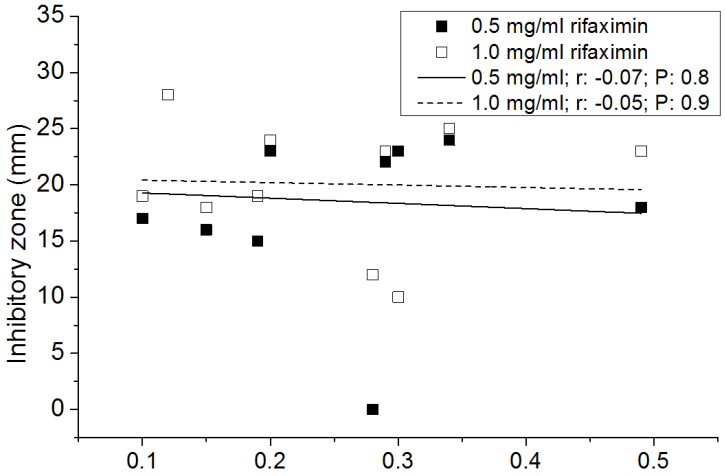

Fig. 5: Relationship between susceptibility of ten isolates of *P. aeruginosa* against 0.5 mg/ml and 1.0 mg/ml of rifaximin in terms of inhibition zone and the biofilm formation of the same ten clinical isolates of *P. aeruginosa* in terms of optical density (OD) at 570 nm. r: Pearson Correlation Coefficient.

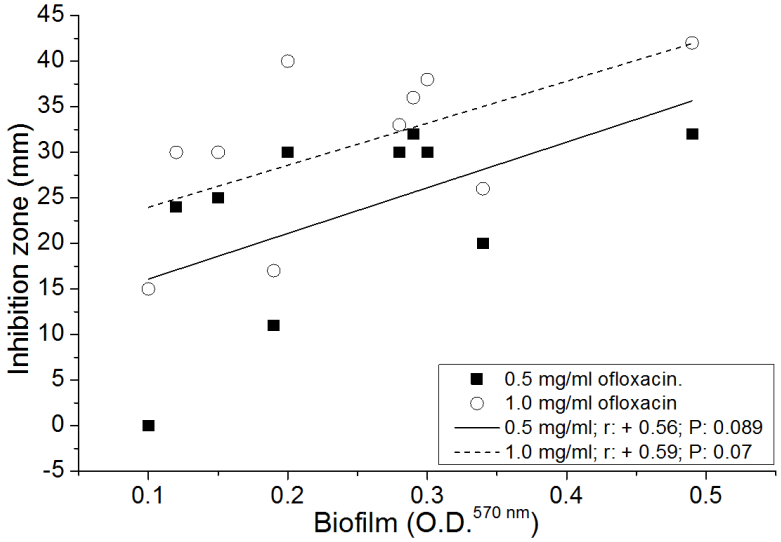

Fig. 6: Relationship between susceptibility of ten isolates of *P. aeruginosa* against 0.5mg/ml and 1.0mg/ml of ofloxacin in terms of inhibition zone and the biofilm formation of the same ten clinical isolates of *P. aeruginosa* in terms of optical density (OD) at 570 nm. r: Pearson Correlation Coefficient.

Bacteria within a biofilm can communicate with each other and coordinate their behavior, making them much more resilient than individual bacteria (Mohammed and Zgair AK (2022).

One of the mechanisms by which biofilms increase antibiotic resistance is by limiting antibiotics' potential to enter the biofilm mass. The EPS matrix acts as a barrier, preventing antibiotics from reaching the bacteria inside. Additionally, biofilm bacteria can undergo genetic changes that make them less susceptible to antibiotics (Sharma *et al*., 2019; Haffiez *et al*., 2023).

The antibacterial effect of rifaximin and ofloxacin was checked using the agar diffusion method. The study showed that the sensitivity of the studied isolates to rifaximin was higher than the sensitivity to rifaximin as it can be noted that the values of the diameters of inhibition zone produced post using 1mg/ml and 0.5mg/ml of ofloxacin against *P. aeruginosa* isolates were higher than the values of the diameters of inhibition zone when using the same concentrations of rifaximin and against the same isolates ($P<0.5$ and $P<0.01$ respectively). The study also showed that all studies isolates formed biofilm *in vitro*. Interestingly, there is no relationship (correlation) between the biofilm formation by studied isolates and their response to the rifaximin. Still, in the case of ofloxacin, a relationship was seen (correlation). Still, it was not statistical, which shows for the first time that resistance to these two antibiotics could be a result of other mechanisms used by bacteria other than the formation of the biofilm. The mechanisms followed by *P. aeruginosa* to resist rifaximin were the low of membrane permeability of bacteria, efflux pump activity and the mutation in RNA (Ribonucleic Acid) Polymerase Beta Subunit (*rpoB* gene) (Langendonk *et al*., 2021; Giovagnorio *et al*., 2023). The other studies showed that the low permeability of the bacterial membrane and the effectivity of the bacterial efflux pump play a crucial mechanism in *P. aeruginosa* resistance to ofloxacin (Langendonk *et al*., 2021; Giovagnorio *et al*., 2023). Moreover, the previous study showed the role of biofilm in the resistance of *P. aeruginosa* to different antibiotics (Fernández-Billon *et al*., 2023).

The previous study showed the relation between the MICs (performed in microtiter plates) of different antibiotics and biofilm formation while in the present study used a different technique (well diffusion method) and two concentrations 1 mg and 0.5mg of rifaximin and ofloxacin to evaluate the relationship between the susceptibility to both antibiotics and ability to form biofilm *in vitro* (Al-Mutalib and Zgair 2023a; Al-Mutalib and Zgair 2023b; Talib and Ghafil, 2024).

This study contributes to opening the way for other studies to know the mechanism by which *P. aeruginosa* is resistant to these two antibiotics. The study of the mechanism of action of rifaximin and ofloxacin against *P. aeruginosa* may contribute to the understanding of the mechanism of resistance of this bacterial species to rifaximin and ofloxacin. Currently, we studied the effect of rifaximin and ofloxacin on the bacterial macromolecules such as nucleic acid, the understanding the ways that followed by bacteria to reduce the effect of the above antibiotics on their will help to indicate the new mechanism may be followed by *P. aeruginosa* to resist rifaximin and ofloxacin.

CONCLUSION

The current study showed that the ability of all clinical isolates of *P. aeruginosa* that used in the present study to form biofilm onto polystyrene *in vitro*. It was shown for the first time that there is no significant relationship ($P>0.05$) between the susceptibility of *P. aeruginosa* to rifaximin and ofloxacin antibiotics, and the biofilm formed by the same bacterial isolates *in vitro*.

ACKNOWLEDGMENTS

This research was funded by Taif University, Saudi Arabia project No. (TU-DSPP-2024-256).

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