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# Antibiotic-Induced Changes in Efflux Transporter Expression: A Key Factor in *Pseudomonas aeruginosa* Biofilm Resistance

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Antibiotic Sensitivity

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**ABSTRACT**

Listed by WHO as an antibiotic-resistant priority pathogen, *Pseudomonas aeruginosa* (P.A.) is a serious threat in nosocomial infections. Its high antibiotic resistance is attributed to major mechanisms that can be categorized into intrinsic, acquired, and adaptive resistance. This study tests the ability of three commonly used antibiotics to inhibit new biofilm formation and eradicate mature biofilm growth, as well as investigate changes in the expression levels of selected genes coding for multidrug efflux pumps in P.A. planktonic cells and biofilms before and after treatment with antibiotics to provide a conceptual estimate of the activity of the efflux transporters that work to extrude antibiotics leading to a reduction in their effectiveness. Antimicrobial susceptibility testing was conducted with Ofloxacin (OFLX), Tobramycin (TOB), and Ceftazidime (CAZ) to determine Mean Inhibitory Concentration (MIC) and Mean Bactericidal Concentration (MBC) using microtiter plate-based biofilm assay and spectrophotometric quantification. Extraction of total RNA was performed from planktonic cultures, inhibition phase, and eradication phase P.A. biofilms. Real-time quantitative reverse transcriptase PCR was utilized to analyze the changes in expression of the *mexAB*, *mexXY*, and *oprM* genes. Three (3) antibiotics that have proven to show less resistance are OFLX, TOB, and CAZ when tested against overnight cultures of P.A. strain PA01. Results showed that OFLX is best for bactericidal properties, which is also supported by the viability assay data obtained from Propidium Iodide staining. Our study showed that the PAO1 strain is susceptible to OFLX for both inhibition and eradication of mature biofilms. TOB was most effective at higher concentrations in the eradication phase.

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## 1 Introduction

*Pseudomonas aeruginosa* is a gram-negative, rod-shaped opportunistic bacterium and one of the most common nosocomial pathogens with high mortality rates (Sousa and Pereira 2014). *Pseudomonas* can easily grow in hospital water systems, humidifiers, and other types of medical equipment such as catheters, ventilators, and pacemakers (Walker and Moore 2014). Further, it can cause both acute and chronic infections as the course of the different infections can vary greatly. Acute infections often involve planktonic bacteria, which are usually treatable with antibiotics, while chronic infections are often difficult to treat, where the bacteria forms into a biofilm. The presence of this organism in biofilm communities exhibits a high degree of Multidrug Resistance (MDR), which makes biofilm-based bacterial infections such as Chronic Obstructive Pulmonary diseases (COPD) which is very challenging to treat. Prevention and treatment strategies are limited by the lack of sensitive detection methods and by the narrow availability of effective antibiotics (Lund-Palau et al. 2016). Thus, in addition to other health complications of *P. aeruginosa*, it contributes to a heavy cost burden on the hospital system.

*P.aeruginosa's* major resistance mechanisms to antibiotics can be classified as either intrinsic, acquired, or adaptive resistance. Intrinsic resistance may be associated with lower outer membrane permeability, expression of efflux pumps, and production of antibiotic-inactivating enzymes. Acquired resistance involves the horizontal transfer of resistance genes or mutational changes, and adaptive resistance may be characterized by the formation of biofilm or multidrug-tolerant cells in the biofilm. The active efflux pumps of antibiotics contribute to the bacterial multidrug-resistant phenotype; therefore, the development of efflux pump inhibitors is a promising adjuvant therapy (Li et al. 2015).

Several studies have shown efflux pumps may play a significant role in the multidrug-resistant phenotype and involvement in the efflux of bacterial factors essential for virulence (Alav et al. 2018). Efflux pumps are membrane proteins that are involved in the export of foreign substances within the bacterial cell. They can pump out a wide range of substrates, including antibiotics, dyes, toxins, waste metabolites, and detergents. The *P.aeruginosa* genome is predicted to encode multiple RNA efflux pumps, four of which are major role players, i.e. MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (Marquez 2005; Poole 2011). MexAB-OprM and MexXY-OprM, the RND-type efflux pumps, account for the major cause of intrinsic resistance to most antimicrobial agents in *P.aeruginosa* (Goli et al. 2018). Both MexXY-OprM and MexAB-OprMare are known to be some of the largest multidrug-resistant efflux pumps within the resistance nodulation division (RND) family in *P.* aeruginosa.

The pumps extrude antimicrobials across the outer membrane, which explains their ability to confer resistance to beta-lactams that target the cell wall synthesis. The pump is comprised of three different peptides, i.e. a MexB translocase belonging to the RND family of solute/proton antiporters, an outer membrane porin-OprM, and a "membrane fusion protein" MexA that docks MexB to OprM. Moreover, the active efflux system MexXY-OprM contributes to aminoglycoside resistance. MexXY-OprM is a multidrug efflux transporter whose specificity is extraordinarily broad but different compared with MexAB-OprM, MexCD-OprJ, MexEF-OprN, and other RND efflux transporters in *P.aeruginosa* (Masuda et al. 2000; Morita et al. 2012). Additionally, the overproduction of the major efflux pumps contributes to the carbapenem resistance in *P. aeruginosa* (Lee and Zhang 2014; Hassuna et al. 2020).

The broad specificity of MDRs seems to match the resistance of biofilms to antimicrobials qualitatively. Biofilms can show very high levels of resistance, and it is unclear whether mechanisms operating in planktonic cells that confer lower levels of resistance play a key role in biofilms. This study utilizes the hypothesis that the deletion of individual pumps or pairs of pumps would make *P.aeruginosa* more susceptible to antibiotic treatment and observe increased activity or mutations (if any) in other pumps quantified by reverse transcription polymerase chain reaction. The objectives of this study were to determine the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of *P.aeruginosa* PA01 biofilm and to elucidate the role of the efflux pumps in antibiotic resistance in biofilms by comparing the expression levels of *mexA*, *mexB*, *mexX*, *mexY*, and *oprM* genes in *P.aeruginosa* planktonic cells and biofilms before and after treatment with antibiotics.

## 2 Materials and Methods

### 2.1 Bacterial strains, growth media, and conditions

The *P. aeruginosa* strain PA01 utilized in this study was preserved by our laboratory at −80°C. Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) media were employed to culture the planktonic form of *P. aeruginosa* PA01 bacteria, whereas M63 media supplemented with arginine, glucose, and casamino acid were used for the formation of PA01 biofilm.

### 2.2 Literature Search

A literature search was conducted from August 2020 to December 2020. Studies on current antibiotic treatment regimens for *P.aeruginosa* and various efflux transporters were collected. Tobramycin (TOB), Ofloxacin (OFLX), and Ceftazidime (CAZ) are promising antibiotics for current *P. aeruginosa* treatment. In addition to a literature search, a Basic Local Alignment Search Tool (BLAST) was utilized, which allowed the finding of regions

with similarity between biological sequences of *mexA*, *mexB*, *mexX*, and *mexY* genes of the PA01 strain by comparing it to select genes of various strains that have been previously studied extensively and are well documented in literature with regards to how they function under different stressors. This is an essential tool for biologists since its efficiency and sensitivity allow scientists to compare nucleotide and protein sequences to both single sequences and large databases to design a primer.

**2.3 Planktonic cell growth**

The planktonic cell growth was measured using a microtiter plate-based assay (Qu et al., 2016). Overnight-grown PA01 cells were diluted 1:100 in L.B. broth. These samples were then grown at 37°C with agitation (160 rpm). The planktonic culture turbidity was read using an Eppendorf spectrophotometer by measuring absorbance at 550nm every hour.

**2.4 Determination of Minimal Inhibition Concentration (MIC)**

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method described by the CLSI 2015. Using McFarlan 0.5 solution as a standard for turbidity of the overnight broth culture, 10 µl of *P. aeruginosa* was dispensed per well in a 96-well plate. The various concentrations of antibiotics were added, and the volume was adjusted with TSB to 100 µl in each well. Each antibiotic (TOB, OFLX, and CAZ) was diluted to a final concentration of 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, and control. The plates were incubated overnight at 37°C. MIC was established as the lowest concentration with the absence of any visible bacterial growth.

**2.5 Determination of Minimal Bacterial Concentration (MBC)**

The MBC of planktonic *P. aeruginosa* was determined for the four antibiotics according to the Manual of Antimicrobial Susceptibility Testing published by the American Society of Microbiology (2005). An overnight PAO1 plate was incubated with 3 loops full of cells in 150 ml of TSB on a shaker at 37°C. Ten-milliliter (10 mL) of culture were taken during the logarithmic phase into 36 sterile tubes (triplicated) and standardized to OD600 before treating with the various concentrations of antibiotics (Ofloxacin: 0.5 µg/ml, 1µg/ml, and 32 µg/ml, Ceftazidime: 4 µg/ml, 8 µg/ml, 16 µg/ml, Tobramycin: 0.5 µg/ml, 1µg/ml, 2 µg/ml). The antibiotic-treated culture was serial diluted $10^{-1}$ to $10^{-7}$ and plated. The plates were incubated at 37°C overnight. CFUs and MBC were established for each plate.

**2.6 Biofilm Eradication and Inhibition Assay**

Newly formed biofilm growth inhibition was analyzed by exposing the culture to antibiotics at the time of inoculation. In contrast, the eradication of mature biofilms was measured by adding antibiotics 24 hours after inoculation, allowing the formation of mature biofilm first, followed by spectrophotometric quantification of the residual biofilm using a modified Microtiter Dish Biofilm Formation Assay (O'Toole 2011).

The biofilm inhibitory assay was carried out in microplates (Qu et al. 2016). 4 µl bacterial suspension from 1: 100 overnight culture and 196 µl TSB was dispensed per well in a 96-well microplate and exposed to different concentrations of the 3 antibiotics as described before, followed by incubation for 24 hours at 37°C. After incubation with antibiotics, the plates were washed with deionized water three times and dried for 4-6 hours. The dried biofilms were stained with a 0.1% solution of crystal violet and incubated at room temperature for 10-15 minutes. Excess stain was removed by rinsing the plate wells three times with deionized water and drying overnight. The residual biofilm biomass in the plate wells was quantified by dissolving the crystal violet attached to the cells in the biofilm in 30% acetic acid and reading the absorbance (O.D. at 550nm) plate reader spectrophotometer.

For biofilm eradication, 100 µl bacterial suspension from 1:100 overnight culture was dispensed per well in microplates. After static incubation at 37°C for 24 hours to allow the formation of a mature biofilm, the supernatants were removed, and the wells were treated with various concentrations of antibiotics. The plates were incubated for 24 hours at 37°C, and biofilm biomass was determined as previously described.

**2.7 Gene expression analysis**

Upon determining the MIC/MBC, concentrations of 8 µg/ml and 32µg/ml of TOB, OFLX, and CAZ were utilized for MIC and MBC, respectively. These concentrations were selected because this is where inhibition (at 8 µg/ml) and eradication (at 32µg/ml) were best observed. Total RNA from the selected concentrations was extracted using RNeasy Mini kit (Qiagen) instructions with RNA protect®Bacteria Reagent added at the required step as indicated in the RNeasy kit instructions. RNA purity and concentration were determined by measuring the absorbance of the sample at 260 and 280 nm (260/280 nm), and 1µL of RNA was used for cDNA synthesis, which was done using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix (Transgene, China).

Expression of the efflux transporter genes was measured by qRT PCR amplification and quantification using the synthesized cDNA. qRTPCR was performed using TransStart™ Green qPCR SuperMix UDG kit (Transgene, China). Conditions for qRT-PCR were the following: 50°C for 2 minutes, initial denaturation at 94°C for 10 minutes, 40 cycles of 5 seconds at 94°C, and 30 seconds at 60°C. The data obtained were normalized to the

endogenous reference gene *pslA* of the *P. aeruginosa* PA01 strain. The threshold cycle method ($2^{-\Delta\Delta CT}$) was used to analyze changes in gene expression in each sample relative to the control, qRT-PCR studies were performed in triplicate using the ProtoScript® First Strand cDNA Synthesis Kit, and the entire experiment was repeated twice with RNA samples extracted from independent cultures.

## 3 Results and Discussion

### 3.1 Selection of primers for gene expression studies

The BLAST tool available on the National Library of Medicine website was utilized to align and compare the DNA sequences of the genes *mexA*, *mexB*, *mexX* and *mexY* (Figure 1), which were selected for further studies on effect of antibiotics on planktonic cells and biofilms formed by the *P. aeruginosa* PA01 strain based on literature search to design the primers needed to facilitate the amplification and quantification of gene expression levels by quantitative real-time polymerase chain reaction (qRTPCR).

The BLAST alignment tool was used to find regions of similarities between the biological sequences of the four efflux transporter genes *mexA*, *mexB*, *mexX* and *mexY*, from *P. aeruginosa* PA01 and that of similar genes from other strains that are documented in the literature. The sequences of the primers used for the qRT PCR-based amplification and quantification of cDNA obtained from the transcribed RNA isolated from the tested samples for analysis of relative expression of these genes, which code for proteins functioning as efflux transporter pumps known to have a potential role in active extrusion of antibiotics from bacterial cells (Bhandari et al. 2022; Lorusso et al. 2022), are listed in Table 1.

The functions of MexA, MexB, MexX, and MexY are primarily transport to structurally varied molecules, including antibiotics out of the bacterial cell. It also functions to lower the intracellular concentration of antibiotics, allowing *P.aeruginosa* to survive at higher antibiotic concentrations, thereby leading to antibiotic resistance (Table 1). In addition, the ribosomal subunit (RpsL) responsible for rRNA and tRNA binding and is a structural constituent of the ribosome is expressed constitutively and therefore

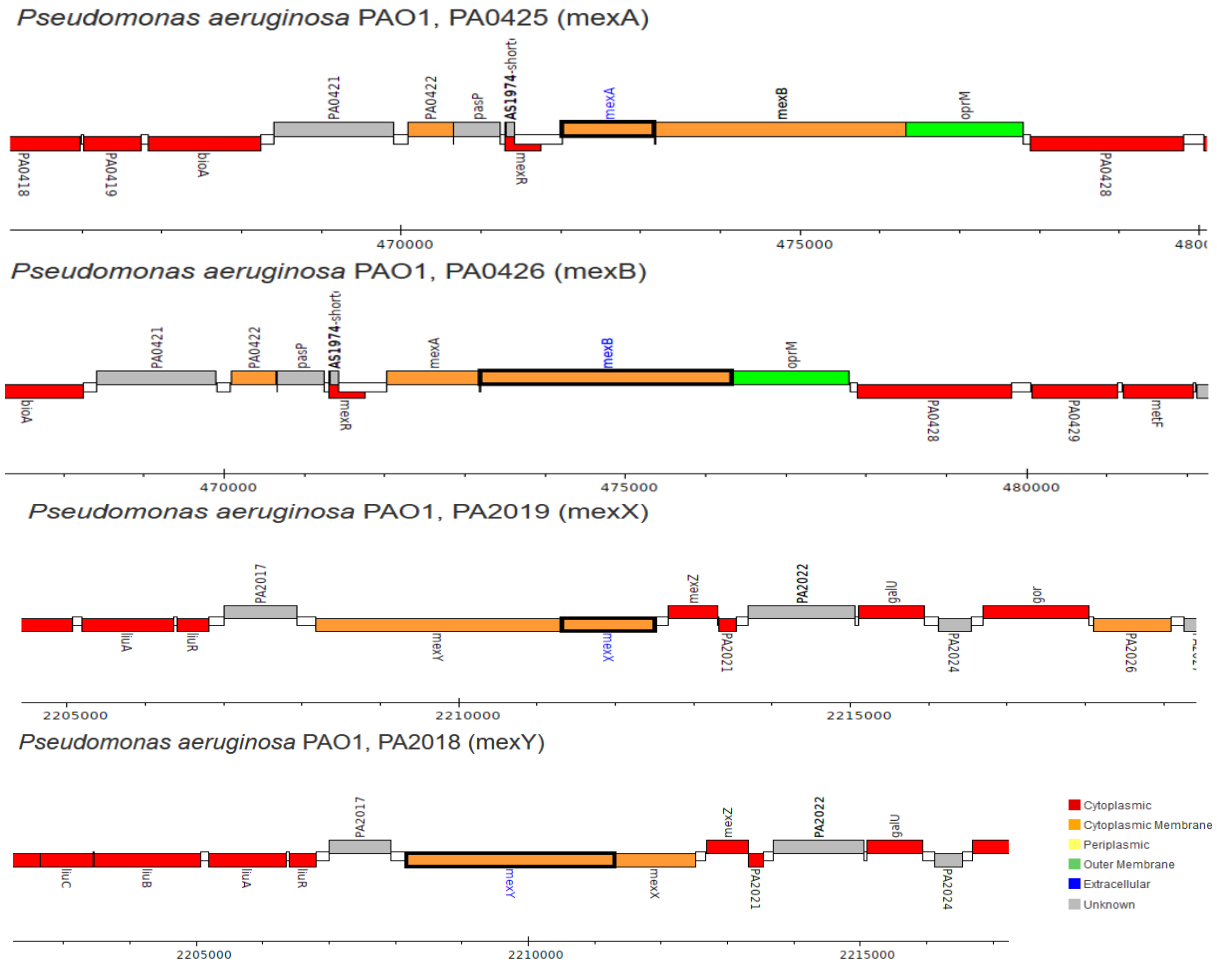


Figure 1 BLAST results for the four main genes responsible for MexA, MexB, MexX, and MexY efflux transporters.

Table 1 Summary of the Functions of Efflux Transporters MexA, MexB, MexX, and MexYin Bacteria.

| Protein | Function | Role in Antibiotic Resistance | Primer Sequence |
|---|---|---|---|
| Multidrug-Resistant Efflux Pump *Mex*A | Resistance Nodulation Cell Division (RND) multidrug efflux Periplasmic membrane fusion protein precursor | Transports structurally varied molecules, including antibiotics, out of the bacterial cell | F: 5'-acctacgaggccgactaccaga-3'<br>R: 5'-gttggtcaccagggcgcctc-3' |
| Multidrug Resistant Efflux Pump *Mex*B | Resistance Nodulation Cell Division (RND) Inner membrane multidrug efflux Transporter protein | Transports structurally varied molecules, including antibiotics, out of the bacterial cell | F: 5'-gtgttcggctcgcagtactc-3'<br>R: 5'-aaccgtcgggattgaccttg-3' |
| Outer Membrane Protein *Opr*M | Major intrinsic multiple antibiotic resistance efflux outer membrane protein OprM precursor | Channel-forming outer membrane protein | F: 5'- ccatgagccgccaactgtc-3'<br>R: 5'-cctggaacgccgtctggat-3' |
| Multidrug Resistant Efflux Pump *Mex*X | Resistance Nodulation Cell Division (RND) multidrug efflux membrane fusion protein Mex X precursor | Transports structurally varied molecules, including antibiotics, out of the bacterial cell | F: 5'-tgtacgcgtattcggaacaaggcgtctgc-3'<br>R: 5'-ttctgctagcgatgtgcatgggtgtccctc-3' |
| Multidrug Resistant Efflux Pump *MexY* | Resistance Nodulation Cell Division (RND) multidrug efflux transporter *MexY* | Transports structurally varied molecules, including antibiotics, out of the bacterial cell | F: 5'-tgtactagttgatgcccctagcgaaactctc-3'<br>R: 5'-tttaagcttgacctacaggacgctgctg-3' |
| Ribosomal Subunit *RpsL* | Ribosomal subunit binding rRNA and tRNA expressed constitutively | Structural constituent of ribosome, which serves as Internal control | F: 5'-gctgcaaaactgcccgcaacg-3'<br>R: 5'-acccgaggtggtccagcgaacc-3' |

utilized as the internal control for evaluating the changes in level of expression of the efflux transporters in response to biofilm formation and presence of various antibiotics (Smith and Iglewski 2003; Rutherford and Bassler 2012; Kishk et al. 2020; Lorusso et al. 2022).

### 3.2 Effectiveness of different antibiotics for inhibition and eradication of *P. aeruginosa* biofilm

The ability of the 3 antibiotics ceftazidime [CAZ], ofloxacin [OFLX] and tobramycin [TOB] to inhibit biofilm formation and eradicate mature biofilms in the *P. aeruginosa* PA01 strain were compared by determination of their Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of the antibiotics. The eradication data compares the effectiveness of these antibiotics on a mature biofilm, which represents clinical biofilm infections. In contrast, the inhibition data compares the effects of the antibiotics mentioned above on a biofilm starting to form from free-living or planktonic cells.

Results presented in Figure 2 revealed the effects of antibiotic application time and concentration on the inhibition of biofilm formation. The antibiotics are applied at the time of inoculation (a) and biofilm eradication (n = 6), where the antibiotic is applied after mature biofilm formation (b) in various concentrations. The x-axis represents the various concentrations of the three antibiotics used, and the y-axis represents the inhibition or eradication of *P. aeruginosa* biofilms obtained because of treatment with the antibiotics as measured by spectrophotometric absorbance at 550 nm to quantify the residual biofilms.

For comparing the effectiveness of the three tested antibiotics for the inhibition of *P. aeruginosa* biofilms, the effect of various concentrations of each antibiotic on the biofilm-forming capacity was assessed using the micro broth format in order to determine the Minimum Inhibitory Concentration (MIC) values which showed dose-dependent sensitivity of PA01 biofilms to both ofloxacin and tobramycin at 8µg/ml concentrations and high resistance to ceftazidime in inhibition phase (Figure 2A). On the other hand, the effectiveness of the three tested antibiotics for eradication of *P. aeruginosa* biofilms were compared by measuring the residual biofilm after incubation of a mature biofilm with various concentrations of each antibiotic to determine the corresponding Minimum Bactericidal Concentration (MBC) values, where a significant resistance to all antibiotics except for ofloxacin was exhibited. In the eradication phase, *P. aeruginosa* biofilms displayed sensitivity to ofloxacin but at much higher concentrations (32 µg/ml) compared to that seen in the inhibition phase, in addition to sensitivity at only the highest concentration of tobramycin (256 µg/ml). The highest level of resistance was observed for ceftazidime, which showed a lack of sensitivity even at the highest concentration, and also displayed significant resistance to tobramycin at lower concentrations, respectively, during the eradication phase (Figure 2B). Antibiotics were applied at the time of inoculation in the 96-well microtiter plate for inhibition, while antibiotics were applied 24 hours after 37°C inoculation for eradication. The optical density of each sample at 550 nm was utilized for collection of data on the inhibition of young biofilms and eradication of mature PA01 biofilms after incubation with increasing concentrations of tobramycin,

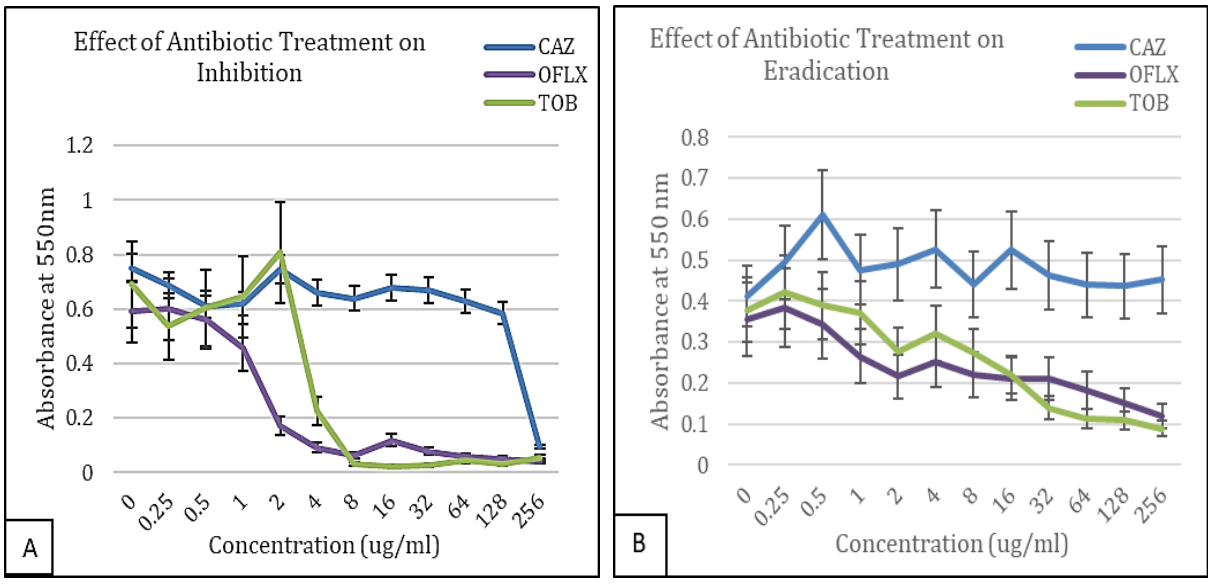


Figure 2 Effect of increasing concentration of the antibiotics ceftazidime[CAZ], ofloxacin [OFLX]and tobramycin [TOB] on (A) inhibition and (B) eradication *of P. aeruginosa* biofilm

ceftazidime, and ofloxacin. Overall, ofloxacin showed the greatest inhibitory and eradicative effect on *P.aeruginosa*, while ceftazidime proved the least effective (Figures 2A and 2B) in both conditions. These observations are corroborated by the data reported by recent studies, which indicated that *P. aeruginosa* biofilms would not be eradicated with low-dose tobramycin (Mangiaterra et al. 2020) and exhibit resistance to beta-lactams, aminoglycosides, fluoroquinones and carbapenems (Zakhour et al. 2022) through processes such as production of carbapenemase, and upregulation of efflux pumps (Hassuna et al. 2020).

### 3.3 Changes in expression of efflux transporter genes in *P. aeruginosa* during biofilm formation

The expression levels of the genes coding for efflux transporters systems MexAB-OprM and Mex XY-OprM were compared in PA01 planktonic cells and biofilms to elucidate changes in their expression in response to factors that trigger the formation of biofilms, which are known to be more resistant than planktonic cells, to inhibition as well as eradication by treatment with antibiotics (Patel et al. 2021).

The expression of the MexAB-OprM and MexXY efflux transporter genes was measured using qRT PCR to compare their expression in planktonic cells and biofilms. All 5 genes showed some degree of upregulation in the biofilm stage as opposed to the planktonic stage (Figure 3), which is also supported by recently published reports showing that a significant majority of antibiotic-resistant clinical isolates of *P. aeruginosa* possessed *mexA* and *mexB* genes which indicates the presence of active efflux-pump system (Bhandari et al. 2022) and their contribution to the higher antibiotic resistance shown by biofilms formed by clinical isolates of *P.aeruginosa* (Hassuna et al. 2020). The isolates possessed mexA and mexB genes, indicating the presence of an active efflux pump system. The *rpsl* gene, which is considered the housekeeping gene, had the same RNA expression levels in both planktonic and biofilm stages.

### 3.4 Effect of various antibiotics on expression of efflux transporter genes in inhibition and eradication phases

The inhibitory and eradicative effects of ceftazidime, ofloxacin and tobramycin on relative expression levels of the MexAB-OprM and Mex XY-OprM transporters system genes were studied in newly formed and mature PA01 biofilms, respectively, by comparing the relative expression level of *mexA, mexB, mexX, mexY* and *oprM* after exposure to the antibiotics at their corresponding MIC values under inhibitory conditions for newly formed biofilms from planktonic cells and MBC values under eradication conditions for mature biofilms to evaluate the possibility that these efflux pumps are involved in the observed decrease in sensitivity to the tested antibiotics during and after biofilm formation.

Figure 4 represents the relative gene expression levels during (A) inhibition of biofilms on treatment with the antibiotics at their determined MIC and (B) eradication of mature biofilms for tobramycin, ceftazidime, and ofloxacin at their determined MBC values. On comparing the effect of the tested antibiotics on the level of expression of *mexA, mexB, mexX, mexY* and *oprM* genes after 24-hour treatment of newly forming and mature PA01 biofilms with each of them at their determined MIC and MBC values, all of the five efflux transporter genes showed significantly increased expression relative to the internal control during both inhibition and eradication phases (Figures 4A and 4B) when

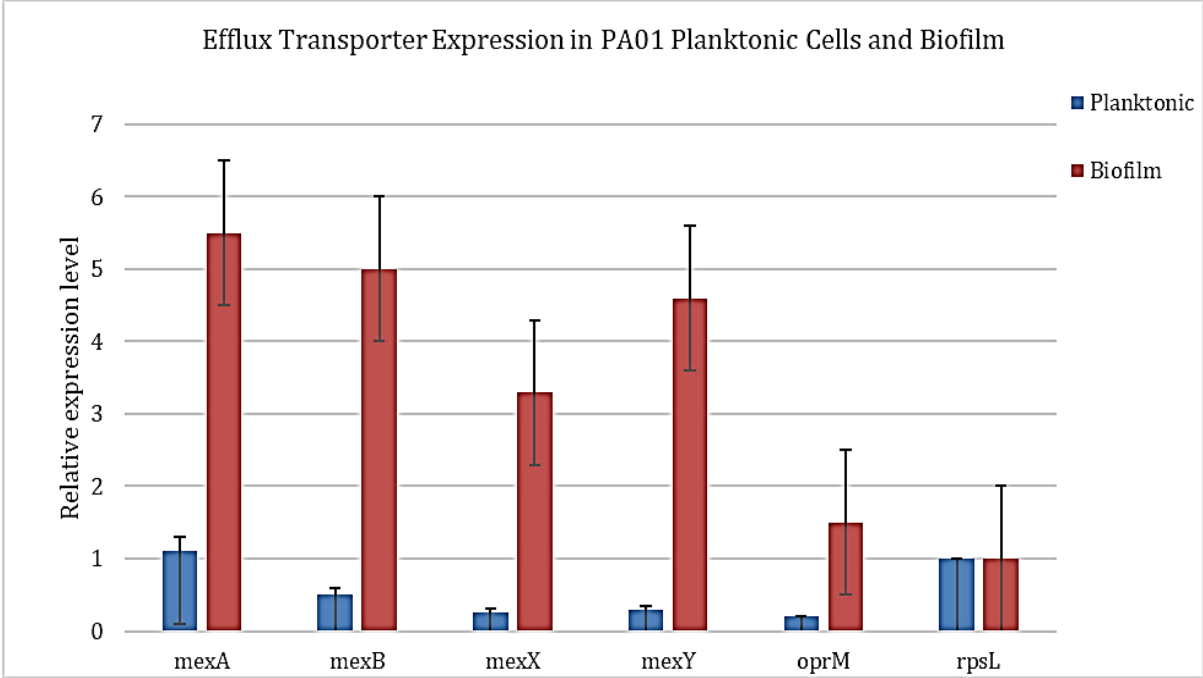


Figure 3 Normalized Expression of Efflux Transporters in PA01 Planktonic Cells and Biofilm; Values represent fold change (mean of triplicate samples) in comparison with the transcription level of the internal control rpsL.

treated with ceftazidime. Earlier studies have reported overexpression of MexAB-OprM systems associated with increased resistance towards cephalosporins (Pourakbari et al. 2016) in *P. aeruginosa* biofilms. On the other hand, treatment with ofloxacin and tobramycin shows a much lesser increase in the expression of *mexA*, *mexB*, *mexX* and *mexY* compared to the expression of *rpsL* during inhibition (Figure 4A) as well as eradication (Figure 4B). This data supports the results obtained on studying the effectiveness of these antibiotics for inhibition and eradication of new and mature *P. aeruginosa* biofilms, which showed that ceftazidime was least effective having the highest MIC and MBC values (Figures 2A and 2B), which could probably be due to the higher expression of the MexAB-OprM and MexXY-OprM efflux pumps leading to increased extrusion of this antibiotic, thus rendering it less useful. Also, both ofloxacin and tobramycin showed comparable effectiveness with similar MIC and MBC values (Figures 2A and 2B), which is backed by the similar level of expression of the efflux pump genes shown after treatment of the PA01 biofilms with ofloxacin and tobramycin under both inhibition and eradication conditions (Figures 4A and 4B).

Empirical antibiotic therapy for suspected cases of *P.aeruginosa* includes monotherapy and combination therapy, such as a β-lactam antibiotic with aminoglycosides. At the same time, the current treatment uses a combination of an antipseudomonal β-lactam (penicillin or cephalosporin) and an aminoglycoside or carbapenem (imipenem or meropenem) with antipseudomonal quinolones with an aminoglycoside. The World Health Organization (WHO) has listed carbapenem-resistant *P.aeruginosa* as one of three bacterial species in critical need of the development of new antibiotics (World Health Organization 2017).

The results reported in this study also show that the efficacy of antibiotics against biofilm formation is time-dependent; the results shown in Figure 2 indicate that less concentration was required to treat an early *P.aeruginosa* infection compared to 24 hours later when a mature biofilm has formed. This study demonstrated that PA01 biofilms are more susceptible to antibiotic treatment in the inhibition phase, as shown by their sensitivity to lower antibiotic concentrations when they are newly forming, compared to the eradication phase when they have matured and only show sensitivity to much higher concentrations. This supports the treatment regimen in monotherapy empirical antibiotic therapy as opposed to combination treatment once the infection is confirmed. Additionally, shifting to a newer combination of antibiotic therapy to treat resistant strains of *P.aeruginosa* may improve outcomes. As *P.aeruginosa* becomes a growing concern, the discovery of antibiotics that the bacteria are not resistant to is significant.

Further genomic analysis was conducted to compare the expression levels of genes coding for proteins that function as efflux pumps, identify those that are overexpressed or suppressed during the antibiotics treatment, and understand the mechanism of PA01 biofilm resistance to the selected antibiotics. With regards to the normalized RNA expression of the efflux transporter genes, it was

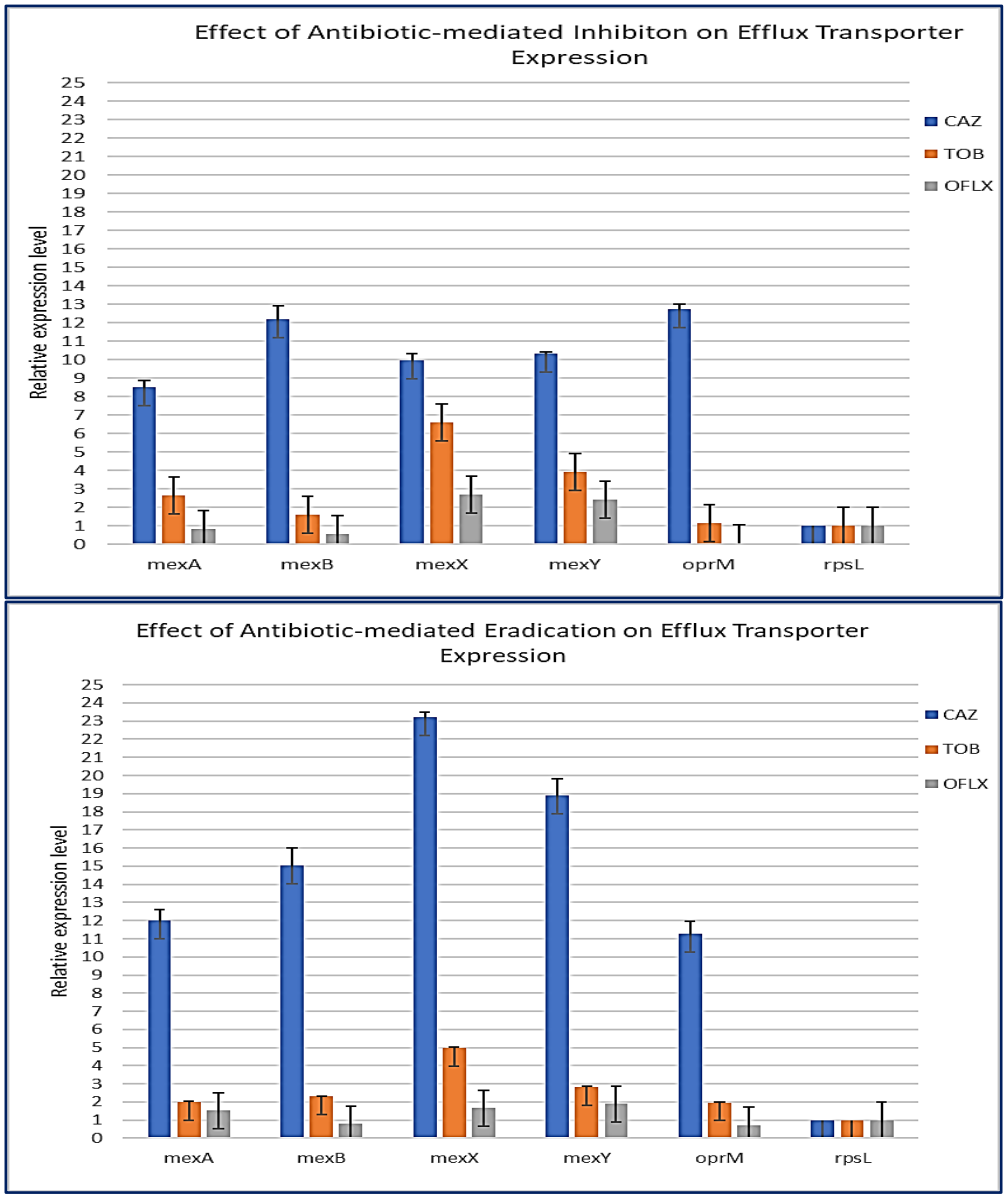


Figure 4 Relative Expression of five Efflux Pump Genes in (*mexA, mexB, mexX, mexY* and *oprM*) PA01 during (A) Inhibition of new biofilm formation and also during (B) Eradication of mature biofilms in the presence of three antibiotics, ceftazidime [CAZ], tobramycin [TOB] and ofloxacin [OFLX] at their Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) respectively as determined previously; Values represent fold change (mean of triplicate samples) in comparison with the transcription level of the internal control *rpsL*

observed that the genes were overexpressed in the biofilm phase as opposed to the planktonic phase (Figure 3). Higher expression of the *mexAB-oprM* and *mexXY-oprM* genes were also observed when PA01 biofilms were treated with ceftazidime compared to ofloxacin and tobramycin as presented in Figure 4. These genes that were upregulated could play a role in lowering the sensitivity to antibiotics by pumping the antibiotics out of the bacterial cells in the biofilm phase. Consequently, they have the potential to act as drug targets for overcoming antibiotic resistance in the future.

## 4 Conclusions

Ofloxacin, tobramycin, and ceftazidime were the most used antibiotics, and they are reported to be effective for the treatment of *P. aeruginosa* infections in this study as well as in the current literature. Out of the three antibiotics tested in both inhibition and eradication phases, ofloxacin [OFLX] was observed to be the most effective since it showed the most inhibitory effect against *P.aeruginosa* strain PA01compared to tobramycin [TOB] and

ceftazidime [CAZ], which showed the least effectiveness. Additionally, both tobramycin and ofloxacin were effective in eradicating biofilm growth. Based on all these results, ofloxacin was the most effective antibiotic for both inhibition and eradication of *P.aeruginosa* biofilms at lower concentrations as opposed to ceftazidime, which was seen to be less effective since the PAO1 strain selected in this study was the most resistant in both MIC and MBC phases.

Furthermore, a comparative analysis of gene expression levels was also done to determine the probable role of the MexAB and MexXY efflux transporters in the observed antibiotic resistance occurring with biofilm formation, which showed higher expression of these genes in PA01 biofilms compared to planktonic cells. Significantly higher expression levels of all the selected efflux pump genes were detected for the treatment of *P. aeruginosa* biofilms with ceftazidime treatment, which was proven to be the least effective, compared to ofloxacin and tobramycin in both inhibition and eradication phases, which may indicate a probable contribution of these efflux pumps in the mechanism of antibiotic resistance associated with these PA01 biofilms.

Deletion studies of both MexA-MexB-OprM and MexX-MexY-OprM efflux pumps also need to be conducted to determine whether they play a significant role in reducing the susceptibility of PAO1 biofilms to antibiotics. This would reduce intrinsic resistance, making *P.aeruginosa* biofilms more sensitive to antibiotics, and reverse acquired resistance could be a promising target for developing new strategies for the treatment of *P. aeruginosa* infections.

Although the efflux pumps play important roles in increasing the resistance towards different antibiotics, the role of other agents and mechanisms in the evolution of resistance should not be ignored. Since the concomitant overproduction of other Mex efflux systems might have additive effects on antibiotic resistance, the co-expressing of a multicomponent efflux pump is recommended. On the other hand, the concomitant overproduction of two Mex pumps might have additive effects on resistance to antibiotics, which would necessitate the co-expression of Mex efflux systems to study their effects. The development of novel antibiotics that can bypass the effects of efflux pumps is still a challenging task. Therefore, further studies on involved mechanisms and structure-function association of bacterial efflux systems, as well as the interactions between the pumps and other resistance mechanisms, are highly recommended.

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**Conflict of interest**

The authors declare that there is no conflict of interest.

**References**

Alav, I., Sutton, J. M., & Rahman, K. M. (2018). Role of bacterial efflux pumps in biofilm formation. *Journal of Antimicrobial Chemotherapy*, *73*(8), 2003-2020. doi:10.1093/jac/dky042

Bhandari, S., Adhikari, S., Karki, D., Chand, A. B., Sapkota, S., et al. (2022). Antibiotic Resistance, Biofilm Formation and Detection of *mexA/mexB* Efflux-Pump Genes Among Clinical Isolates of *Pseudomonas aeruginosa* in a Tertiary Care Hospital, Nepal. *Frontiers in Tropical Diseases, 17*(2), 2021. https://doi.org/10.3389/fitd.2021.810863

Cavalieri, S. J. (2009). Manual of Antimicrobial Susceptibility Testing. United States: American Society for Microbiology.

Goli, H. R., Nahaei, M. R., Rezaee, M. A., Hasani, A., Kafil, H. S., et al. (2018). Role of MexAB-OprM and MexXY-OprM efflux pumps and class 1 integrons in resistance to antibiotics in burn and intensive care unit isolates of pseudomonas *aeruginosa*. *Journal of Infection and Public Health, 11*(3), 364-372. doi:10.1016/j.jiph.2017.09.016

Hassuna, N. A., Darwish, M. K., Sayed, M., & Ibrahem, R. A. (2020). Molecular epidemiology and mechanisms of high-level resistance to meropenem and imipenem in *Pseudomonas aeruginosa*. *Infection and Drug Resistance*, *13*, 285-293. doi:10.2147/IDR.S233808

Kishk, R.M., Abdalla, M.O., Hashish, A.A., Nemr, N.A., El Nahhas, N., et al. (2020). Efflux MexAB-Mediated Resistance in *P. aeruginosa* Isolated from Patients with Healthcare Associated Infections. *Pathogens 9*(6), 471. https://doi.org/10.3390/ pathogens9060471

Lorusso, A.B., Carrara, J.A., Barroso, C.D.N., Tuon, F.F., & Faoro, H. (2022). Role of Efflux Pumps on

Antimicrobial Resistance in *Pseudomonas aeruginosa*. *International Journal of Molecular Sciences, 23*, 15779. https://doi.org/10.3390/ijms232415779

Lee, J., & Zhang, L. (2014). The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein & Cell*, *6*(1), 26-41. doi:10.1007/s13238-014-0100-x

Li, X., Plésiat, P., & Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in gram-negative bacteria. *Clinical Microbiology Reviews*, *28*(2), 337-418. doi:10.1128/CMR.00117-14

Lund-Palau, H., Turnbull, A. R., Bush, A., Bardin, E., Cameron, L., et al. (2016). *Pseudomonas aeruginosa infection in cystic fibrosis: Pathophysiological mechanisms and therapeutic approaches* Informa U.K. Limited. doi:10.1080/17476348.2016.1177460

Mangiaterra, G., Cedraro, N., Vaiasicca, S., Citterio, B., Galeazzi, R., et al. (2020). Role of Tobramycin in the Induction and Maintenance of Viable but Non-Culturable *Pseudomonas aeruginosa* in an In Vitro Biofilm Model. *Antibiotics*, *9*(7), 399. https://doi.org/10.3390/antibiotics9070399.

Marquez, B. (2005). Bacterial efflux systems and efflux pumps inhibitors. *Biochimie*, *87*(12), 1137-1147. doi:10.1016/j.biochi.2005.04.012

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., & Nishino, T. (2000). Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, *44*(9), 2242-2246. doi:10.1128/AAC.44.9.2242-2246.2000

Morita, Y., Tomida, J., & Kawamura, Y. (2012). MexXY multidrug efflux system of *Pseudomonas aeruginosa*. *Frontiers in Microbiology*, *3*, 408. doi:10.3389/fmicb.2012.00408

O'Toole G. A. (2011). Microtiter dish biofilm formation assay. *Journal of visualized experiments*, *47*, 2437. https://doi.org/10.3791/2437

Patel, D., Sen, P., Hlaing, Y., Boadu, M., Saadeh, B., & Basu, P. (2021). Antimicrobial Resistance in *Pseudomonas aeruginosa* Biofilms. *Journal of Pure and Applied Microbiology*,*15*(4):2520-2528.

Poole, K. (2011). Pseudomonas *aeruginosa*: Resistance to the max. *Frontiers in Microbiology*, *2*, 65. doi:10.3389/fmicb.2011.00065

Pourakbari, B., Yaslianifard, S., Yaslianifard, S., Mahmoudi, S., Keshavarz-Valian, S., & Mamishi, S. (2016). Evaluation of efflux pumps gene expression in resistant Pseudomonas aeruginosa isolates in an Iranian referral hospital. *Iranian journal of microbiology*, *8*(4), 249–256.

Qu, L., She, P., Wang, Y., Liu, F., Zhang, D., et al. (2016). Effects of norspermidine on pseudomonas *aeruginosa* biofilm formation and eradication. *Microbiology Open (Weinheim)*, *5*(3), 402-412.

Rutherford, S. T., & Bassler, B. L. (2012). Bacterial quorum sensing: Its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in Medicine*, *2*(11), a012427.

Smith, R. S., & Iglewski, B. H. (2003). *Pseudomonas aeruginosa* quorum sensing as a potential antimicrobial target. *The Journal of Clinical Investigation*, *112*(10), 1460-1465. doi:10.1172/JCI200320364

Sousa, A. M., & Pereira, M. O. (2014). *Pseudomonas aeruginosa* diversification during infection development in cystic fibrosis Lungs—A review. *Pathogens (Basel)*, *3*(3), 680-703.

Walker, J., & Moore, G. (2014). *Pseudomonas aeruginosa* in hospital water systems: Biofilms, guidelines, and practicalities. *The Journal of Hospital Infection*, *89*(4), 324-327. doi:10.1016/j.jhin.2014.11.019

World health organization. (2017). Retrieved from https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

Zakhour, J., Sharara, S.L., Hindy, J.R., Haddad, S.F., & Kanj, S.S. (2022). Antimicrobial Treatment of *Pseudomonas aeruginosa* Severe Sepsis. *Antibiotics*, *11*(10), 1432.