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Detection of *MexR* and *MexZ* efflux pump genes and assessment of the synergistic effect of amikacin and gentamycin with some efflux pump inhibitors in antibiotic-resistant *Pseudomonas aeruginosa*

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Abstract

Background: A bacterium called *Pseudomonas aeruginosa* causes infections with high mortality rates and has the ability to rapidly acquire resistance to a variety of antibiotics.

Methods: A total of 118 clinical specimens from both males and females of different age groups were gathered between early September 2022 and the end of December 2022 from several hospitals in Al-Anbar city, Iraq. The sample sources were wounds, burns, urinary tract infections (UTI), and sputum. *P. aeruginosa* was identified through its microscopical properties, confirmed using the VITEK 2 automated system, followed by molecular detection using the 16S rRNA gene. The Kirby-Bauer disk diffusion method was used based on CLSI guidelines (CLSI, 2022) for the aminoglycoside antibiotics class. DNA extraction from the twenty isolates was conducted using Presto™ Mini gDNA Bacteria Kit. After that, molecular analysis of *MexR* and *MexZ* was performed using the PCR technique.

Results: Within this, 65 isolates were determined as *P. aeruginosa* from 118 samples. The proportion of *P. aeruginosa* isolates from wound samples was 27/65 (41.5%), while in the burn wound, Urinary tract infection (UTI), and sputum were 20/65 (30.8%), 14/65(21.5%), and 4/65(6.2%), respectively. The findings of the test for antibiotic resistance were Amikacin (73.84%), Gentamycin (81.53%), Netilmicin (33.84%), and Tobramycin (66.15%). The molecular analysis of *MexZ* genes from the twenty isolates showed the highest prevalence, 20/20 (100%), and the *MexR* gene 14/20 (70%). The antagonism of aminoglycoside antibiotics by divalent cations was found, and it is noted that the presence of divalent cations increased the antibiotic Minimum Inhibitory Concentration (MIC) values.

Conclusion: It was found that *MexR* and *MexZ* genes are widespread in *P. aeruginosa* isolates, which indicated elevated *mexXY-OprM* and *MexAB-OprM* gene expression in *P. aeruginosa*. These elevated efflux pumps of *P. aeruginosa* may influence antibiotic ion fluxes at the bacterial cell membrane, contributing to the improved resistance to aminoglycosides. The aminoglycoside resistance isolates expressed decreased efflux pumps when inhibited by CCCP and Conessine.

Introduction

Pseudomonas aeruginosa is a bacterium from the family "Pseudomonadaceae". Due to the risk to hospitalized patients, the mortality rate, antibiotic resistance, and production of extracellular toxins, this bacterium is among the most significant species [1]. Additionally, it contains significant virulence characteristics that compromise immune protection and result in substantial tissue damage [2]. This bacterium has intrinsic resistance to several types of antibiotics, leading to infections that are challenging to treat and that have a high morbidity and fatality rate [3]. Aminoglycosides are broad-spectrum antibiotics that gained prominence in the early 1940s with the discovery of streptomycin from a *Streptomyces* species [4]. Since then, at least ten aminoglycosides have been approved for clinical usage, with amikacin, gentamicin, netilmicin, and tobramycin being the most often used [5]. Reduced outer membrane permeability, active efflux pumps, and enzymes that alter aminoglycosides are the systems by which *P. aeruginosa* develop resistance to these antibiotics [6]. Efflux pump transport proteins play a role in extruding harmful substrates from inside cells to the external environment, thereby lowering intracellular antibiotic concentrations [7]. Antibiotic efflux resistance, which is accomplished by drug efflux pumps, is an important microbial resistance mechanism. Because the drug excretion rate is generally quicker than the drug penetration rate, the drug level in the cell may be regulated to a non-sensitive level [8]. *P. aeruginosa* has a higher prevalence of four of these efflux pumps: Resistance to aminoglycosides, beta-lactams, and fluoroquinolones can be caused by *MexXY-OprM*; resistance to fluoroquinolones can be caused by *MexEF-OprN*; and resistance to beta-lactams and fluoroquinolones can be caused by *MexCD-OprJ* and *MexAB-OprM* [9]. The *MexXY* operon, controlled by the *MexZ*, encodes *MexXY* components. *MexXY* is an inducible antibacterial. The induction of this system can be triggered by ribosome disruption [10]. *MexAB-OprM* expression is controlled by *MexR*, which inhibits the synthesis of *MexAB-OprM* [11]. A specific antibiotic causes bacteria with a mutant *MexR* gene to produce more *MexA*, *MexB*, and *OprM* proteins, which in turn causes efflux pumps to be overexpressed and antibiotic resistance to rise [12].

Efflux pump inhibitors (EPIs) are compounds that block efflux pumps through several methods [13]. Carbonylcyanide m-chlorophenylhydrazone (CCCP) is a well-known laboratory efflux pump inhibitor. It can be a potent inhibitor of RND EPs. It is a potent uncoupling agent that can stop the efflux pump from working by reducing the transmembrane electrochemical gradient and suppressing the chemical synthesis source, which

in turn lowers the uptake of aminoglycosides by *P. aeruginosa* and lowers their bactericidal activity [14]. As well as conessine, it is a steroid alkaloid derived from the plant *Holarrhena antidysenterica*. It was isolated from a plant species in the Apocynaceae family. It possesses antimicrobial properties by inhibiting *P. aeruginosa MexAB-OprM* [15]. Indeed, in a number of *P. aeruginosa* cases of resistance to aminoglycosides in hospitals, the development of the resistant genes encoded by these antibiotics is a major problem. The current study was designed to study the resistance of *P. aeruginosa* to aminoglycosides, and also investigated the genetic basis of efflux pump-mediated aminoglycoside resistance in *Pseudomonas aeruginosa* isolated from clinical specimens through the detection of some antibiotic resistance-related genes, *MexR*, and *MexZ* in some isolated strains. Moreover, the study of the antibacterial activity of some aminoglycoside antibiotics and their synergistic interactions with efflux pump inhibitors, conessine, and carbonyl cyanide-m-chlorophenylhydrazone, against the isolated strains was also studied.

Methods

Sample collection and identification

A total of 118 clinical specimens from both males and females of different ages were gathered from the start of September 2022 to the end of December 2022, from patients at distinct province hospitals of Al-Anbar governorate, Iraq. The sample sources were wounds, burns, urinary tract infections (UTI), and sputum. The *P. aeruginosa* was identified through its cultural and microscopical properties [16] and confirmed by the pattern of biochemical profiles using the VITEK 2 automated system, followed by molecular detection using the *16S rRNA* gene.

Antimicrobial Susceptibility Tests

The aminoglycoside antibiotics discs were selected: Amikacin, Gentamycin, Tobramycin, and Netilmicin. The diffusion method was applied to assess resistance for the bacterial isolates according to (CLSI-2022): A few colonies were added to brain-heart broth (2 mL) and cultured to obtain the bacterial suspension together with McFarland turbidity. A cotton swab was used to culture the bacterial suspension into Muller-Hinton agar plates. Antimicrobial discs were positioned on the medium's surface, then incubated at 37°C for 24 hours. The diameter of each antibiotic disk's inhibitory zone was measured and contrasted with the usual inhibition zone of CLSI-2022.

DNA isolation and molecular analysis

Following the guidelines provided by the manufacturer, genomic DNA was isolated from bacterial culture using

DNA isolation kits (Geneaid, Korea). A Nanodrop device was used to measure the purity and concentration of the DNA, which was then stored at -20 °C to avoid deterioration. Molecular weight markers (100 bp), 1X (TAE) buffer, and 1% agarose gel were manufactured following the guidelines provided by the manufacturer. The sequences for the Mex R and Mex Z genes were verified by using the Nucleotide Blast program of NCBI. In the end, oligonucleotides of Reverse and forward primers synthesized by the BIONEER company (Korea), as described in Table 1, were selected.

Gene	Primer sequence (5'-3')	Size (bp)	References
<i>MexR</i>	F- CGCCATGGCCCATATTGAG	637bp	Osman <i>et al.</i> , [17]
	R- GGCATTGCGCAGTAAGCGG		
<i>MexZ</i>	F- AGCGGCGCGACAGTAGCATA	781bp	Osman <i>et al.</i> , [17]
	R- CCGAGGACCGCGCAGGC		

Table 1: Oligonucleotide primers and their information.

*R: the Reverse sequence F: the Forward sequence

Preparation of PCR mixture

A PCR reaction of 25 µl was made of green master mix (Promega), template DNA, primer solution, and deionized water.

Determination of minimum inhibitor concentration (MIC)

The Resazurin Microtitre Plate Assay (REMA) was used to assess the antibiotic solutions. 100 µl Mueller-Hinton broth was poured into each plate well under aseptic conditions. Next, 100 µl of the test substance was moved into the 96-well plates' first row. To do serial dilutions, 100 µl of the material was pipetted from the first row to the next rows in progressively decreasing concentrations as (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256). Ten microliters of bacterial suspension (1.5×10^8 CFU/mL) were added to each well [18].

Synergism between Carbonyl cyanide m-chlorophenylhydrazine and Conessine with aminoglycoside antibiotics: Checkerboard assay

To determine if two test materials combined in microtiter plates had an antibacterial impact, a checkerboard assay was employed. Each well of the initial microtiter plates received 100 µl of Mueller-Hinton broth (MHB) to maintain consistency with the MIC determinations. Next, 100 µL of the first test material was poured into the initial column, moving it vertically to the next wells and discarding the remaining 100 µL. To obtain a concentration in serial form 1/2 into 1/128, the second test substance (100 µL) was placed into the first row of a different microtitre plate, and another 100 µL was moved horizontally into the next wells, discarding the remaining 100 µL. Each concentration on the first microtitre plate receives a vertical transfer of the second material dilutions using

a pipette. Each well received 10 µL of bacterial suspension (1.5×10^8 CFU/mL). To prevent contamination, after being covered with Parafilm, the plates were incubated at 37°C for a full day. Following incubation, 100 µl of Resazurin was added to each well, and the plate was subsequently incubated again for 2–6 hours before results were read. The well that did not change color was then regarded as the intersection of the MICs of the two antibacterials used together. Following the calculation of each antibiotic's fractional inhibitory concentration (FIC), the Fractional Inhibitory Concentration Index (FICI) was computed. The FICI is most frequently used to define or characterize drug interactions.

The FIC for two antibacterials (B and A) working together is as follows:

$$FIC(A) = \frac{MIC(A) \text{ combination with antibiotic (B)}}{MIC(A) \text{ alone}}$$

A: is the first test material, and B: is the second test material.

Σ FIC(FICI) is therefore given by:

$$FICI = FIC(A) + FIC(B)$$

A FICI value of ≤ 0.5 is indicative of a synergistic effect. FICI values > 0.5-4 are indicative of an additive effect (no drug interaction). Moreover, FICI > 4 indicates an antagonistic effect.

Synergy

A combination's MIC is at least two dilutions lower than the MIC of an antibiotic used alone, and its results are noticeably better than the sum of its independent activity when assessed separately.

Additive

When two medications are taken together, their effects are identical to those of the combination (the equally effective concentrations) when taken separately.

Antagonism

The combination's MIC is at least two dilutions higher than the MIC of the antimicrobial alone, and the combined result is noticeably worse than the best individual result [12].

Results

Isolation of *P. aeruginosa* and identification

In this study, the collected samples were taken four months from the start of September until the end of December 2022. From 118 clinical specimens, 65 isolates were identified as *P. aeruginosa*, because of the microscopic examination that showed the *P. aeruginosa* colony, which has been colored pink, referred to as gram-negative, rod-shaped, non-spore-forming, single bacteria or arranged in pairs.

Isolate	Antibiotic	Antimicrobial	antibiotic MIC (µg/mL) By REMA	Antimicrobial MIC (µg/mL) By REMA	Combined Antimicrobial MIC	Combined antibiotic MIC	FICI (ΣFIC)	Outcome
1	Amikacin	Conessine	31.2	50	1.56	7.81	0.21	Synergistic
1	Amikacin	CCCP	31.2	12.5	0.78	3.91	0.18	Synergistic
1	Gentamycin	Conessine	2.5	50	1.56	0.62	0.27	Synergistic
1	Gentamycin	CCCP	2.5	25	0.78	0.31	0.15	Synergistic

Table 2: Synergism between antibiotics and Antimicrobials (conessine, CCCP) against *P. aeruginosa*.

To confirm the results, the isolates were diagnosed utilizing the (VITEK-2 compact system), and the Polymerase chain reaction (PCR) technique was used for confirmatory identification of *P. aeruginosa* isolates by detecting 16S rRNA gene. These results showed that the 65 isolates were *P. aeruginosa*. This study revealed that most of the isolates (41.5%) were found in wound samples, whereas the percentage of isolates from burn samples was 30.8%, Sputum (6.2%), and UTI (21.5%).

Antimicrobial Susceptibility Test

The disk diffusion method of Kirby-Bauer was applied to assess the antibiotic sensitivity of 65 *P. aeruginosa* samples. In accordance with (CLSI, 2022), susceptibility was tested toward the aminoglycoside antibiotics class, including amikacin, gentamycin, tobramycin, and netilmicin. The percentage of *P. aeruginosa* isolates shows high levels of resistance: 53/65 (81.53%) for Gentamycin, 48/65 (73.84%) for Amikacin, 43/65 (66.15%) for Tobramycin, and 22/65 (33.84%) for Netilmicin.

Molecular analysis

The molecular study for 20 isolates showed the percentage of the *MexR* gene was 14/20(70%), with a size product (637 bp) as shown in Figure 1. The results revealed that 20/20 (100%) isolates had the *MexZ* gene, as shown in Figure 2.

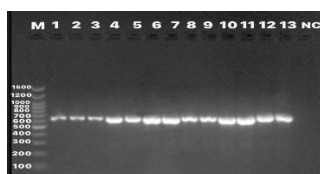


Figure 1: The fragments of the Uniplex PCR amplification to detect the *MexR* gene (637 bp). Ladder of M:100 bp DNA. Amplicon was electrophoresed on a 1.5% agarose gel at 70 V for 1.5 hours, stained with ethidium bromide, and seen using the documentation system for UV transilluminators.

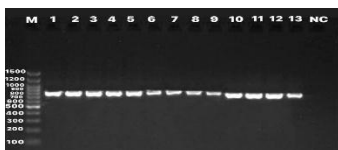


Figure 2: The fragments from Uniplex PCR amplification to identify the *MexZ* gene (781 bp). Ladder of M:100 bp DNA. A UV transilluminator's documentation system was used to observe the amplicon after it had been electrophoresed on an agarose gel (1.5%) for 70 V/cm for 1.5 hours and stained with ethidium bromide.

Determination of minimum inhibitor concentration by using Resazurin Microtitre-plate Assay (REMA) method

The results of MIC to the aminoglycoside group were: Amikacin (31.2 µg/mL) and Gentamycin (2.5 µg/mL) for both conessine and CCCP.

Evaluation of the effect of efflux pump inhibitors and an antibiotic solution combination using the checkerboard technique

The Checkerboard assay of *P. aeruginosa* gave synergistic profiles when efflux pump inhibitors (Conessine, CCCP) were combined with aminoglycoside antibiotics, separately (Table 2 & Figure 3). Minimum Inhibitory Concentration (MIC) of Amikacin alone for the test isolate was 31.2 µg/mL. When combined with the inhibitors, efficiency increased considerably. In the presence of conessine, the amikacin MIC decreased 4-fold from 31.2 to 7.81 µg/mL; with CCCP, it decreased 8-fold to 3.91 µg/mL. The inhibitors' MICs in combination were 1.56 µg/mL (conessine) and 0.78 µg/mL (CCCP), respectively (Table 2). Since all FICI values were below 0.5, synergism is present, and as the number approaches zero, the degree of synergy increases.

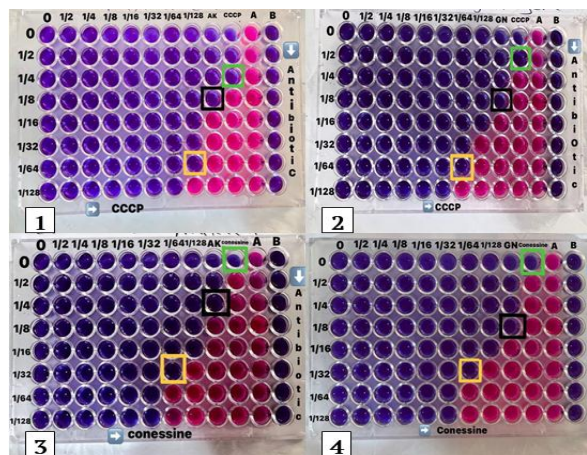


Figure 3: Checkerboard assay showing antimicrobial combinations: (1) Amikacin (AK) with CCCP, (2) Gentamycin (GN) with CCCP, (3) Amikacin (AK) with conessine, and (4) Gentamycin (GN) with conessine. The numbers on the left and at the top indicate dilution numbers from 1/2 to 1/128 for each test material, as listed by the arrow. The wells represented by yellow squares indicate the point at which the MIC of the combination materials lies. The wells represented by black squares indicate the point at which the MIC of the antibiotic is. (A) positive control (media + bacterial growth), (B) negative control (media)

Discussion

In the findings of the current study, the highest percentage of *P. aeruginosa* in clinical isolates belongs to wounds, 27/65 (41.5%), followed by burns, 20/65 (30.8%). Kamali *et al.* [19] found that 10% of isolates that were obtained from the wound infection were *P. aeruginosa*. These results differed from our results. Another study, consisting of 235 different specimens in Baghdad city, revealed a percentage of clinical isolates (36%) of burns and (16%) of wound infection [20]. Due to their compromised immune system, prolonged hospitalization, and therapeutic and diagnostic procedures, burn patients are more likely than other patients to get infections. Furthermore, *P. aeruginosa* contamination of hospital air, equipment, and personnel's hands may be the origin of a significant portion of burn infections [21]. *P. aeruginosa* is a nosocomial infection that can occur during the implantation and removal of indwelling urinary catheters and contribute to urinary tract infections [22]. In critically sick pneumonia patients, admission to an intensive care unit (ICU) increases the possibility of *P. aeruginosa* infection [23]. A bacterium, *P. aeruginosa*, has shown resistance to the aminoglycoside group of antibiotics. Aminoglycoside resistance is mediated in *P. aeruginosa* via reduced outer membrane permeability, aminoglycoside modification enzymes, and an active efflux pump [6]. The percentage of the *MexR* gene found was 70% [24]. Also, the percentage of the *MexR* gene was found to be 79% [25]. Efflux pumps enable microorganisms to expel internal poisons, such as antibiotics. One of the largest MDR of efflux pumps, the *MexAB-OprM* pump is significantly expressed in *P. aeruginosa* and is regulated through the regulatory gene *MexR*. *MexA*, *MexB*, and *OprM* protein synthesis increase in response to antibiotic exposure in bacteria with the mutant *MexR* gene, which promotes the overexpression of efflux pumps and increases antibiotic resistance [23].

The results showed that the *MexZ* gene was detected in our study at a percentage of 100%, which indicates that the gene is widespread in isolates resistant to aminoglycosides. The *MexZ* repressor protein is often considered a key factor in the resistance of aminoglycosides in *P. aeruginosa*. Clinical isolates of *P. aeruginosa* frequently include *MexZ* mutations, which are linked to increased *mexXY* expression [26]. The most prevalent route by which *P. aeruginosa* imparts resistance to aminoglycosides is *MexXY-OprM* expression [27].

Patients who are bacteremic and have already been exposed to antibiotics may benefit from an antibiotic-inhibitor combo therapy [28,29]. Among all the tactics used to address the issue of resistance to antibiotics, surveillance of resistance to antibiotics is crucial.

Alternative methods of dealing with infections caused by drug-resistant bacteria become necessary as a result of the rise in bacterial resistance to antibiotics and the dearth of newly available treatments [30]. Developing antibiotic substitutes and finding or creating adjuvants are two possible approaches that have been suggested [31]. Some attempts have been undertaken to boost or restore antimicrobial activity against germs that are resistant to multiple drugs [32]. When chemical inhibitors are added to antibiotics, the antibacterial MIC may decrease [33,34]. Finding ways to increase the antibiotics' diffusion over bacterial membranes or to block the efflux pumps, which is a common resistance mechanism in Gram-negative bacteria, is essential in increasing the antibiotics' effectiveness [35].

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Author Contributions

The authors shared the development, material preparation, and collection of the samples. Every author has read and approved the finished manuscript.

Competing Interest

No conflicts of interest are disclosed by the authors.

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