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Research Article

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Detection of possible aminoglycosides resistance mechanisms in *Pseudomonas aeruginosa* resistant isolates

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Abstract

Globally, there is a growing concern about *Pseudomonas aeruginosa* resistance to aminoglycosides. Enzymatic modification of these drugs is the predominant resistance mechanism. Additionally, *P. aeruginosa* possesses many efflux systems that enable it to resist a variety of antimicrobial agents. This study aimed to determine the resistance patterns to several antibiotics as well as the possible mechanisms of aminoglycosides resistance, including aminoglycosides modifying enzymes (AMEs), genes, and active efflux system, observed in clinical *P. aeruginosa* isolates recovered from patients admitted to Minia University Hospitals, Minia, Egypt. Isolates of *P. aeruginosa* were identified by traditional phenotypic tests and assessed for their *in vitro* susceptibility to various antibiotics. The minimum inhibitory concentrations (MICs) of some aminoglycosides were determined without and after the addition of carbonyl cyanide m-chlorophenylhydrazone (CCCP). Aminoglycoside resistance amplified gene sequences were detected using polymerase chain reaction (PCR). Antibiotic sensitivity testing was applied on 93 clinical isolates of *P. aeruginosa*. The highest rate of resistance was recorded against cefepime and ceftazidime (94.6 % each), while 35.5 % of the examined strains exhibited resistance to minimally one of the evaluated aminoglycoside antibiotics. Furthermore, 49.5 % of isolates were multidrug-resistant (MDR). After CCCP addition, 24.2 % of the resistant isolates restored their sensitivity to gentamicin. According to PCR analysis, *aac(3)-II* was the most frequently detected gene (21.2 %) followed by *aph(3')-VI* (15.2 %), and *aac(6')-IIa* (3 %). Multiple drug resistance was observed among *P. aeruginosa* strains included in this study. Resistance of *P. aeruginosa* to aminoglycosides is greatly influenced by efflux pumps. Coordinated measures and further investigations are urgently needed to manage aminoglycosides resistance.

Keywords: *Pseudomonas aeruginosa*, Multi-drug resistance, Aminoglycosides resistance, Efflux pump inhibitors

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative rod-shaped aerobic bacterium, which is extensively dispersed in nature, but is more prevalent in health care settings. The distinctive characteristics of *P. aeruginosa* strains that promote their survival in medical facilities include its capacity to develop resistance to various antibiotics and endure stressful conditions, including temperature changes, high salt concentration, and antiseptics (Imanah *et al*., 2017). This bacterium is one of the main pathogens implicated in hospital-acquired infections (HAI), including surgical wounds, urinary tract infection, bacteremia, endocarditis, pneumonia, cystic fibrosis, and otitis media (Ghanem *et al*., 2023; Panwar *et al*., 2024), and is the main cause of death in burned individuals worldwide (Al-Charrakh *et al*., 2016).

Aminoglycosides are highly effective bactericidal antibiotics that are frequently prescribed to treat serious infections caused by both Gram-positive and Gram-negative bacteria (Al-Jubori *et al*., 2015; Alshammari *et al*., 2019). These agents inhibit microbial proteins synthesis by binding to the A-site on the 16S ribosomal RNA within the 30S ribosomal subunit making it unavailable for translation leading to cell death (Krause *et al*., 2016). Aminoglycosides are still an effective therapy for *P. aeruginosa* infections and express synergy with other antibiotics such as β-lactams, thus facilitate cell death and lower the risk of developing drug resistance (Rossolini and Mantengoli, 2005).

Increased bacterial resistance to different aminoglycosides such as gentamicin, tobramycin, and amikacin, has been recorded in recent years among nosocomial pathogens all over the world (Alshammari *et al*., 2019; El-Far *et al*., 2021). Numerous factors contribute to the widespread of aminoglycosides resistance; however their excessive use in treatment of serious infections is the primary factor that affected their therapeutic efficacy (Saipriya *et al*., 2018).

Aminoglycoside resistance can occur through inactivation of the drug by aminoglycosides modifying enzymes (AMEs), decreased outer membrane permeability, active efflux system, and alteration of the ribosomal target location (Garneau-Tsodikova and Labby, 2016). However, enzymatic inactivation is considered as the most frequent mechanism (Smith *et al*., 2017) that prevents the aminoglycosides' attachment to the bacterial cell ribosome (Rossolini and Mantengoli, 2005).Three classes of AMEs were identified, including aminoglycoside phosphotransferases (APHs), acetyltransferases (AACs), and nucleotidyltransferases (ANTs) (Smith *et al*., 2017). The most prevalent modifying enzyme genes in *P. aeruginosa* are *aph(3')*, *aac(6')-I*, *aac(6')-II*, and *ant(2'')-I* and their substrates, which represent the most significant anti-pseudomonal aminoglycosides (Vaziri *et al*., 2011).

Efflux pump inhibitors (EPIs) lower the resistance levels and increase intracellular concentration of antimicrobial drugs, which are crucial factors for success of treatment, but their toxicity is the major challenging problem (Fanélus and Desrosiers, 2013). One of the commonly used *in-vitro* EPIs is carbonyl cyanide m-chlorophenylhydrazone (CCCP). It decreases ATP synthesis through its oxidative phosphorylation activity and promotes permeability of the cell membrane by disrupting the proton motive forces (Jeong *et al*., 2016).

The objectives of the study were to determine the aminoglycoside resistance rate among *P. aeruginosa* stains recovered from different clinical samples and explore the prevalence of *aph(3')-VI*, *aac(6')-IIa*, and *aac(3)-II* genes using PCR. In addition, we

investigated how CCCP affected the MICs of some aminoglycosides.

2. Materials and methods

2.1. Isolation and clinical identification

From July 2021 to March 2022, 245 clinical samples were obtained from individuals receiving care at Minia University Hospitals and suffering from various infections; mainly wound infections (70 samples), otitis media (51 ear discharge samples), urinary tract (40 urine samples), respiratory tract (20 sputum samples and 19 tracheal aspirates), bacteremia (30 blood samples), and eye infections (10 pus swabs). These specimens were cultured initially on Cetrimide agar (HiMedia, India), used as a selective medium for *P. aeruginosa* isolation, and MacConkey agar (HiMedia, India) employed as a selective medium for Gram negative bacteria and to detect the microorganism's ability to ferment lactose. All inoculated plates were incubated for 24 h at 37 °C (Ezeador *et al*., 2020). *P. aeruginosa* isolates were identified using different biochemical assays such as sugar fermentation test, oxidase test, and blue green pigment production (Cheesbrough, 2006). Isolates of *P. aeruginosa* were kept until processing at -80 °C in glycerol stocks. *P. aeruginosa* ATCC27853 (Thermo-Scientific™, USA) was used as a positive control.

2.2. Antibiotic susceptibility testing

Following the criteria approved by the Clinical and Laboratory Standards Institute (CLSI. 2023), the antibiotic susceptibility of all *P. aeruginosa* isolates was evaluated using the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar (MHA) plates (HiMedia, India) against 13 antibiotics, including piperacillin/tazobactam (100/10 µg), aztreonam (30 µg), ceftazidime (30 µg), cefepime (30 µg), meropenem (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), norfloxacin (10 µg), ofloxacin (5µg), gentamicin (10 µg), amikacin (30 µg), and tobramycin (10 µg) (Oxoid, Basinstoke, UK). The antibiotic susceptibility assay was conducted two times in triplicates. Isolates that showed resistance to any of the tested aminoglycosides; mainly amikacin, gentamicin, and/or tobramycin were defined as aminoglycoside resistant. Multidrug-resistant (MDR) phenotype was assigned to an isolate that showed no susceptibility to at least one tested antibiotic of the three or more antibiotic classes (Magiorakos *et al*., 2012). The minimum inhibitory concentrations (MICs) of tobramycin, gentamicin, and amikacin (EPICO, Egypt) of the aminoglycoside resistant isolates were further assessed using the agar dilution method on MHA plates in accordance with CLSI breakpoints (CLSI. 2023). During this assay, *P. aeruginosa* ATCC27853 was a quality control strain used according to CLSI protocol.

2.3. Treatment with an efflux pump inhibitor

For the resistant *P. aeruginosa* isolates, gentamicin, tobramycin, and amikacin MICs were detected again as described above but after the addition of CCCP (Sigma-Aldrich Ltd, USA) to each MHA plate at a final concentration of 6.25 µg/ ml. The assay was run in triplicates. Additional positive control plate containing EPI and a negative control plate inoculated with a bacterium only without antibiotic were used. Antibiotic resistance attributable to an efflux mechanism was detected by at least four fold declines in MIC of each antibiotic upon addition of CCCP (Azimi *et al*., 2016).

2.4. Detection of genes encoding aminoglycosides modifying enzymes by polymerase chain reaction

Genomic DNA from pure colonies of resistant *P. aeruginosa* strains was extracted using boiling method. Briefly, the bacterial isolates were cultured aerobically on nutrient agar (NA) at 37 °C for 18-24 h. In sterile 1.5 ml tubes, three to six colonies were picked from the plates and mixed with 0.1 ml dnase/rnase-free water to make a turbid suspension of bacteria ($1\text{-}2\times10^9$ cells/ ml). These suspensions were placed for 10 min. in a boiling water-bath to lyse the cells then centrifuged at 10,000 g for 10 min. at 4 °C. Finally, the supernatant was transferred into another sterile tube and kept at -20 °C before its use as a template in PCR

reaction (Adabi *et al*., 2015). Aminoglycoside resistant isolates were screened for the existence of three different AMEs genes, including *aph(3')-VI*, *aac(6')-IIa*, and *aac(3)-II*, using conventional PCR. The sequences of primer pairs employed in the current study and specific annealing temperatures for each reaction are illustrated in Table (1). PCR amplification was carried out by adding template DNA (2 µl) to a PCR mixture comprising of 1 µl of each primer (10 pM/ µl) (forward and reverse), 12.5 µl of 2x PCR ready-made Master mix. (COSMO PCR Hot Start Master Mix, willowfort, UK), and 8.5 µl nuclease-free water (Hazra *et al*., 2019). Each PCR product was separated on 1 % agarose gel and stained with 0.5 µl/ ml ethidium bromide. Amplicon sizes were determined using ready-to-use 100 bp Plus DNA marker (Thermo-Scientific™, USA), which was run with each gel.

2.5. Statistical analysis

The Statistical Package of Social Science (SPSS) program version 22 (Chicago, USA) was employed for data statistical analysis. The Z- score test was used to compare proportions. *P* value < 0.05 indicated that the result was statistically significant.

Table 1: Primer sequences utilized in this study and conditions conducted for each PCR reaction

Primer	Sequence (5′-3′)	Annealing temperature (°C)	Size (bp)	References
aph(3')-VI	F:ATGGAATTGCCCAATATTATT	55 °C	780	(Aishwarya *et al*., 2020)
	R:TCAATTCAATTCATCAAGTTT			
aac(6')-IIa	F:CCATAACTCTTCGCCTCATG	52 °C	542	(Kashfi *et al*., 2017)
	R:GAGTTGTTAGGCAACACCGC			
aac(3)-II	F:ATATCGCGATGCATACGCGG	55 °C	877	(Asghar and Ahmed, 2018)
	R:GACGGCCTCTAACCGGAAGG			

3. Results

3.1. *P. aeruginosa* isolation and identification

In our study, a total of 93 (37.9 %) isolates of *P. aeruginosa* were obtained from 245 various clinical samples. *P. aeruginosa* was a Gram negative and oxidase positive bacterium that showed non-lactose fermenting growth on MacConkey agar and a characteristic blue-green pigment (pyocyanin) on cetrimide agar. The results revealed higher frequency of *P. aeruginosa* in males [n = 58 (62.4 %)] than in females [n = 35 (37.6 %)]. The prevalence of *P. aeruginosa* isolates in relation to different specimen types is displayed in Table (2).

3.2. Antimicrobial susceptibility testing

Table (3) illustrates the antimicrobial profile of several antibiotics used for treatment of *P. aeruginosa*

Table 2: Prevalence of *P. aeruginosa* isolates in different types of clinical specimens

Type of samples	Number of samples	*P. aeruginosa* isolates No. (%)
Urine	40	15(37 %)
Wound	70	21(30 %)
Sputum	25	16(64 %)
Ear discharge	51	20(39.2 %)
Blood	30	8(62.7 %)
Tracheal aspirates	19	9(47.4 %)
Eye discharges	10	4(40 %)
Total	245	93(37.9 %)

Where; Percent (%) is related to the number of specimens of the same type

Table 3: Antimicrobial susceptibility profile of *P. aeruginosa* isolates

Antibiotics	No. (%)		
	Sensitive	Intermediate	Resistant
Cefepime	5(5.4 %)	0 (0 %)	88 (94.6 %)
Ceftazidime	5(5.4 %)	0 (0 %)	88 (94.6 %)
Imipenem	42(45.2 %)	1(1.1 %)	50(53.8 %)
Meropenem	59(63.4 %)	0 (0 %)	34(36.6 %)
Aztreonam	51(54.8 %)	6(6.5 %)	36(38.7 %)
Ciprofloxacin	60(64.5 %)	3(3.2 %)	30(32.3 %)
Levofloxacin	64(68.8 %)	0(0 %)	29(31.2 %)
Norfloxacin	62(66.6 %)	1(1.1 %)	30(32.3 %)
Ofloxacin	63(67.7 %)	0(0 %)	30(32.3 %)
Amikacin	63(67.7 %)	1(1.1 %)	29(31.2 %)

Gentamicin	60(64.5 %)	0 (0 %)	33(35.5%)
Tobramycin	66 (71 %)	3(3.2 %)	24(25.8 %)
Piperacillin/tazobactam	58(62.4 %)	8(8.6 %)	27(29 %)

Where; Percent (%) is correlated to the total number of *P. aeruginosa* isolates

infections. Among all isolates, 46/93(49.5 %) were recorded as MDR. High resistance rates were detected against both cefepime and ceftazidime (n = 88, 94.6 % each) and were followed by imipenem (n = 50, 53.8 %). The same levels of resistance were detected against ciprofloxacin, norfloxacin, and ofloxacin (n=30, 32.3 % each). In this study, 35.5 % (33/93) of the isolated *P. aeruginosa* exhibited resistance against one or more of the examined aminoglycoside antibiotics.

3.3. Minimum inhibitory concentration with efflux pump inhibitor

Minimum inhibitory concentration analysis of some aminoglycosides antibiotics was performed with and without carbonyl cyanide m-chlorophenylhydrazone (CCCP) against resistant *P. aeruginosa* isolates to confirm the efflux pump role in bacterial resistance to this group of antibiotics. Table (4) displays the role of CCCP in reducing MICs of the tested antibiotics. After CCCP addition, a four-fold reduction or more in each antibiotic MIC value was observed indicating an active efflux pump, and gentamicin was effluxed by 20 *P. aeruginosa* isolates (60.6 %). Our results revealed that MICs of gentamicin, tobramycin, and amikacin decreased in (23/33, 69.7 %), (8/24, 33.3 %), and (9/29, 31 %) of the isolates, respectively. Moreover, 24.2 % and 13.8 % of the resistant isolates regained their sensitivity to gentamicin and amikacin; respectively, after adding CCCP, while 12.5 % of the isolates reverted to intermediate tobramycin MIC breakpoint. The number of isolates that restored their susceptibility to the tested aminoglycosides was recorded statistically as very highly significant (*P*= 0.0001). Addition of CCCP had no impact on the MIC of amikacin in 69 % of the isolates and on tobramycin MIC in 66.7 % of the isolates (Table 5).

3.4. Detection of genes encoding for aminoglycosides modifying enzymes

Using PCR analysis, results revealed that among 33 resistant isolates, 13(39.4 %) of them carried AMEs-encoding genes ; the most prevalent one was *aac(3)-II*, identified in 7 isolates (21.2 %), while the others were *aph(3')-VI* and *aac(6)-IIa*, with frequencies of 5(15.2 %) and 1(3 %), respectively. No coexistence of genes was observed among the tested isolates. Fig.'s (1, 2, and 3) demonstrate the PCR amplification bands of *aph(3')-VI*, *aac(3)-II*, and *aac(6')-IIa* genes; respectively, which were detected in resistant *P. aeruginosa* isolates.

4. Discussion

P. aeruginosa has emerged as a significant pathogen, accounting for 10-15 % of life-threatening health care associate infections worldwide (Strateva and Yordanov, 2009). Out of 245 clinical samples collected in this study, 93 (37.9 %) isolates of *P. aeruginosa* were detected. This finding was in accordance with those obtained from other Egyptian studies (Mohamed *et al*., 2019; Ghanem *et al*., 2023) and lower than that revealed by El-Badawy *et al*., (2019) (90.2 %) and Ahmed *et al*., (2020) (67 %). However, lower prevalence of *P. aeruginosa* infections was reported by Gill *et al*., (2016) from India, Mirzaei *et al*., (2020) from Iran, and Mohamed *et al*., (2022) from Egypt. The relative diversity of *P. aeruginosa* incidence rates may be significantly influenced by the geographic, climatic, and hygienic factors (Ahmed *et al*., 2020).

Table 4: Aminoglycosides MICs for resistant *P. aeruginosa* isolates with and without carbonyl cyanide m-chlorophenylhydrazone (CCCP) addition

Strain no.	Gentamicin		Tobramycin		Amikacin	
	MIC_1	MIC_2	MIC_1	MIC_2	MIC_1	MIC_2
	(µg/ ml)					
P. aeruginosa ATCC27853	**0.5**	**0.5**	**0.5**	**0.5**	**2**	**2**
1	256	**16**	S	ND*	64	64
2	256	**16**	S	ND*	64	64
3	16	**2**	128	**8**	64	64
4	256	**2**	128	128	64	64
5	>1024	**1**	1024	**4**	S	ND*
6	1024	512	1024	512	1024	1024
7	256	**4**	64	**8**	64	64
8	32	32	S	ND*	S	ND*
9	64	**16**	256	128	S	ND*
10	32	32	32	32	64	64
11	128	**8**	32	32	128	128
12	32	32	32	32	64	32
13	64	**2**	32	32	256	**8**
14	64	**8**	256	128	128	128
15	128	**16**	64	64	64	64
16	512	512	64	64	64	64
17	256	**64**	128	128	64	64
18	128	**16**	S	ND*	S	ND*
19	256	256	S	ND*	64	64
20	32	**8**	256	256	128	128
21	128	128	256	256	512	512
22	64	64	512	512	64	**8**
23	256	**64**	S	ND*	64	64
24	64	32	32	32	128	128
26	64	**2**	S	ND*	64	**8**
27	128	128	32	32	128	**32**
28	64	32	64	64	128	64
29	32	32	S	ND*	64	64
30	256	**2**	32	**8**	64	64
31	16	16	32	32	64	32
32	256	**2**	128	**4**	64	**16**
33	512	**32**	S	ND*	256	**64**
34	128	**32**	64	64	64	64

Where; ND*: not determined, S: sensitive; MIC1: MIC of an antibiotic alone, MIC2: MIC of an antibiotic in presence of CCCP. Bold numbers indicate ≥ 4 fold reduction in MICs of the examined antibiotics

Table 5: Impact of CCCP addition on MIC breakpoints of resistant *P. aeruginosa* isolates against different tested antibiotics

MIC reduction	Isolates tested for gentamicin MIC n=33	Isolates tested for tobramycin MIC n=24 No (%)	Isolates tested for amikacin MIC n=29
No MIC reduction	10(30.3 %)	16(66.7 %)	20(69 %)
MIC reduction above susceptible breakpoint	12(36.4 %)	3(12.5 %)	2(6.9 %)
MIC reduction at or below susceptible breakpoint	8(24.2 %)	2(8.3 %)	4(13.8 %)
MIC reduction to intermediate breakpoint	3(9.1 %)	3(12.5 %)	3(10.3 %)
P value	0.0001*	0.0001*	0.0001*

Where; Percent (%) correlated to the number of isolates resistant to each antibiotic, *P-value < 0.05 by Chi-square test was significant

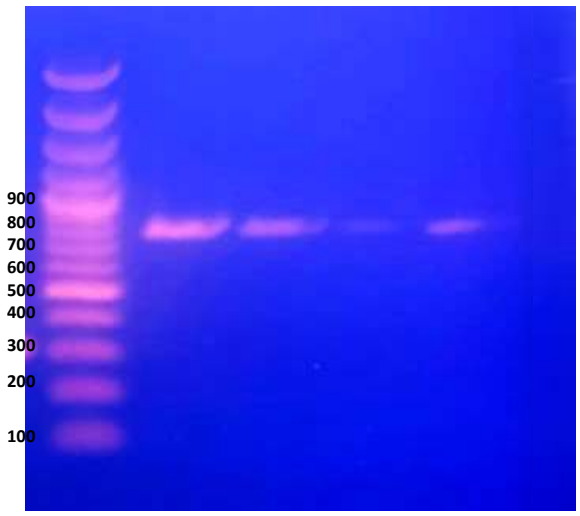

Fig. 1. PCR amplified product of *aph(3')-VI* gene (780 bp). L: 100 bp plus DNA ladder; P: positive control (780 bp); N: negative control; 1, 2 and 3 lanes represent positive isolates

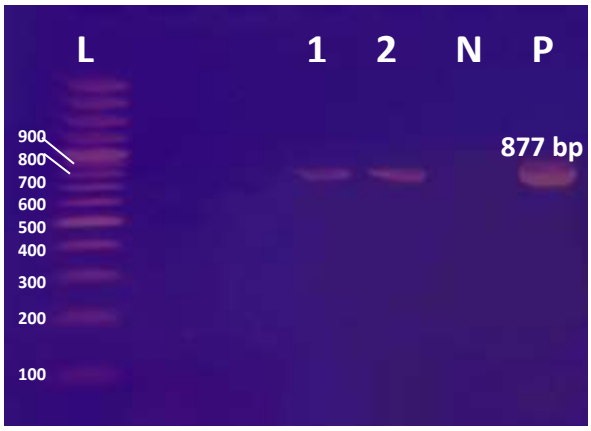

Fig. 2. PCR amplified product of *aac(3)-II* gene (877 bp). L: 100 bp plus DNA ladder; P: positive control (877 bp); N: negative control; 1 and 2 represent positive isolates

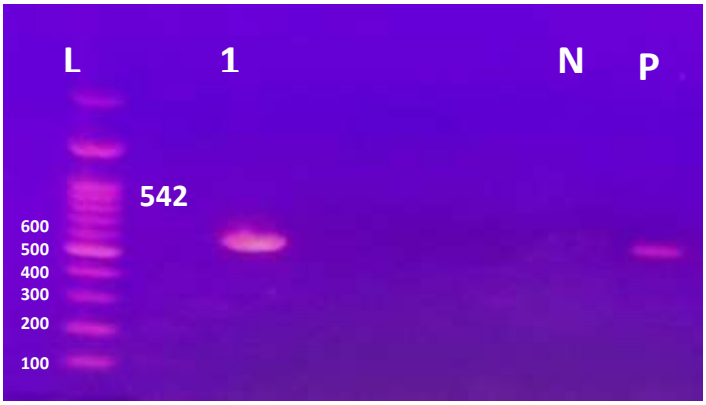

Fig. 3. PCR amplified product of *aac(6')-IIa* gene (542 bp). L: 100 bp plus DNA ladder; P: positive control (542 bp); N: negative control; 1: represents a positive isolate

According to the type of samples, our data showed high prevalence of *P. aeruginosa* in sputum samples (64 %), followed by blood samples (62.7 %), tracheal aspirates (47.4 %), eye discharges (40 %), ear discharges (39.2 %), urine samples (37 %), and finally wound exudates that showed the least incidence (30 %). Likewise, Ahmadian *et al*., (2021) observed the prevalence of *P. aeruginosa* among sputum samples but at lower frequency than ours (37 %). Regarding wound exudates, our results were in agreement with those reported previously in Egypt by Raouf *et al*., (2018); Elmaraghy *et al*., (2019); Hassuna *et al*., (2020). Different results were detected by El-Far *et al*., (2021) who recorded the prevalence of *P. aeruginosa* in urine samples (66.7 %), followed by sputum (12.1 %), pus (15.1 %), and blood (6 %). Another study reported by Hazra *et al*., (2019) has detected lower frequency of *P. aeruginosa* in various clinical

specimens, including pus (34.29 %), blood (18.57 %), tracheal aspirates (17.85 %), and urine (11.43 %). Reduced rates of *P. aeruginosa* infections were also recorded in Iran by Nouri *et al*., (2016) among different types of samples.

Treatment of *P. aeruginosa* infections is hampered by the inherent resistance of this species, along with its capacity to develop resistance against different antibiotic classes (Bassetti *et al*., 2018). *P. aeruginosa* strains investigated in this study exhibited remarkable resistance rates, as verified by other studies conducted in Egypt by Abbas *et al*., (2018); Okasha, (2021), which call for the development of a national antibiotic policy as several previous global studies recorded significant lower MDR rates. The percentage of MDR isolates in our research was 49.5 %, which is lower than that obtained by Ghanem *et al*., (2023); Gondal *et al*., (2024), but greater than the rate implied recorded by El-Far *et al*., (2021). In a Mexican survey of Ochoa *et al*., (2015), over half of *P. aeruginosa* strains were MDR, which is consistent with this study.

Over the past decade, resistance to 3rd and 4th generation cephalosporins in Egypt revealed an alarming ascending pattern, as noticed in several previous setting's (Ahmed *et al*., 2020; Elbrolosy *et al*., 2020; Okasha, 2021). Much lower results were obtained from a study conducted in Venezuela, where resistances of 17.5 % and 23.4 % were observed to cefepime and ceftazidime, respectively (Teixeira *et al*., 2016). In this study, 53.8 % of isolates were carbapenem resistant while 32.3 % expressed resistance to fluoroquinolones. These rates were more than those reported by Abbas *et al*., (2018) but lower than antibiotic resistance values recorded in several other studies reported by Pérez *et al*., (2019); Zahedi Bialvaei *et al*., (2021).

Aminoglycosides are highly effective broad-spectrum antibiotics used widely in treating *P. aeruginosa* serious infections (Labby and Garneau-Tsodikova, 2013). However, the advent of resistant bacteria decreased the efficacy of aminoglycosides in empiric therapies (Sultan *et al*., 2018). In the current study, 35.5 %, 31.2 %, and 25.8 % of the isolated *P. aeruginosa* were respectively resistant to gentamicin, amikacin, and tobramycin. This observation was consistent with another Egyptian study reported by Henrichfreise *et al*., (2007) but higher than the results of another study carried out in Spain by Delgado *et al*., (2007). Additionally, prevalence of aminoglycoside resistance observed in our study was less than that recorded in several other previous studies (Hassuna *et al*., 2020; El-Far *et al*., 2021; Afify *et al*., 2024). Conversely, Basha *et al*., (2020) observed high rates of resistance to gentamicin (70 %) and amikacin (66 %). Updating our knowledge concerning resistance characteristics of *P. aeruginosa* in accordance with time and geographical changes worldwide is crucial to restore the use of a variety of aminoglycoside as an anti-pseudomonal drug (El-Far and Abukhatwah, 2023).

Efflux pumps inhibition is anticipated to reduce the level of inherent drug resistance. Combining EPIs with an antibiotic is a promising strategy for enhancing the therapeutic effectiveness of these drugs. Moreover, EPIs may be used in different phenotypic tests to detect the acquired drug resistance mediated by efflux pump overexpression (Zavascki *et al*., 2010).

Regarding the phenotypic impact of EPI on aminoglycosides resistant isolates, our results displayed 2 ≥32 fold decline in MICs of the investigated antibiotics after incorporation of CCCP, which are in corroboration with another study that has recorded the same folds of reduction in gentamicin MIC against *P. aeruginosa* isolates (Talebi-Taher *et al*., 2016). According to Rajamohan *et al*., (2010), adding CCCP significantly lowered the MICs of several biocides from two to twelve times, while Azimi *et al*., (2016) reported 4-1024 folds reduction in gentamicin MIC used with CCCP against *P. aeruginosa* obtained from burn and non-burn people.

Our investigation revealed that 24.2 % of resistant isolates reinstated their susceptibility to gentamicin after the addition of CCCP. Comparable findings were reported by Adabi *et al*., (2015); Talebi-Taher *et al*.,

(2016) who reported that resistant isolates of *P. aeruginosa* were more susceptible to different antibiotics in the presence of CCCP, with ratio ranging from 25 % to 51 %. These tested antibiotics included cefepime, ciprofloxacin, imipenem, and gentamicin.

According to the results observed in the previous studies conducted by Okasha, (2021); Zahedi Bialvaei *et al*., (2021), many *P. aeruginosa* isolates displayed increased sensitivity to aminoglycosides and fluoroquinolones after incorporation of Phe-Arg β-naphthylamide dihydrochloride (PaβN) as EPI. These results validated that efflux pumps play a great role in development of resistance to the commonly used anti-pseudomonal agents (Azimi *et al*., 2016).

Gram-negative bacteria have most frequently developed resistance to aminoglycosides through enzymatic inactivation (Teixeira *et al*., 2016). In the present work, *aac(3)II* was the most frequent aminoglycoside resistance gene, which was detected in 21.2 % of the resistant strains, followed by *aph(3')-VI* (15.2 %) and *aac(6')-IIa* genes (3 %). These observations were convenient with a previous Saudi investigation, which recorded that *aph(3')-VI* and *aac(6')-II* genes were found correspondingly in 16.7 % and 5.5 % of the resistant strains (El-Far and Abukhatwah, 2023). In an Indian study, prevalence of *aac(3)II* genes was 8.57 % (Hazra *et al*., 2019), while Asghar and Ahmed, (2018) from Saudi Arabia found that none of the tested *P. aeruginosa* harbored this gene. Furthermore, the frequencies of *aph(3')-VI* gene in the previous studies reported by Park, (2009); Vaziri *et al*., (2011) are compatible with results of this study, as their percentage reached 14.8 % and 11 %, respectively. Recently, Saeli *et al*., (2024) reported the presence of *aph(3')-VI* gene in 3.1 % of clinical *P. aeruginosa* isolates in Iran. In another Iranian study, prevalence of *aac(6')-II* gene is higher than ours (10 %) (Kashfi *et al*., 2017), in contrast to the outcomes of another French study conducted by Dubois *et al*., (2008) where relatively lower incidence of the same gene has been detected (1.9 %).

Our results revealed that 39.4 % of resistant isolates harbored only a single AME gene in contrast to other studies that revealed coexistence of more than one resistance genes in *P. aeruginosa* isolates (Al-Jubori *et al*., 2015; Teixeira *et al*., 2016; Alshammari *et al*., 2019). Additionally 60.6 % of our isolates were devoid of any types of AMEs genes despite their resistance to at least one of the investigated aminoglycoside antibiotics. Thus, *P. aeruginosa* resistance may be attributed to other resistance mechanisms. Finally, more genetic data is needed for implementation of novel treatment approaches in addition to infection prevention and quality control procedures (El-Far and Abukhatwah, 2023).

Conclusion

Although aminoglycosides are still effective anti-pseudomonal treatments, resistance to these antibiotics remains a serious issue, particularly in Egypt. Our results confirmed the crucial role of efflux pump systems in the emergence of MDR among *P. aeruginosa* clinical isolates. Furthermore, existence of aminoglycoside resistance genes on mobile genetic elements promotes their spread and dissemination among the other bacteria. Therefore, continuous local surveillance of aminoglycoside resistance is essential. More concerted efforts and additional research studies are required to reduce aminoglycosides resistance prior to becoming a life-threatening issue.

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Conflict of interest

The authors declare no conflicts of interest in this study.

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Ethical approval

The research procedures were ethically approved by the Ethics Committee, Faculty of Pharmacy, Minia University, Minia. Egypt (No. 230603). Informed consents were obtained from all participants involved in this study.

Author's Contributions

Conceptualization, M.R.B., R.A.I. and N.G.F.M.W.; Roles/Writing-original draft, M.R.B. and N.G.F.M.W.; Data curation, M.R.B., R.A.I., N.A.H. and N.G.F.M.W.; Formal analysis, G.F.M.G. and N.A.H.; Investigation, M.R.B., R.A.I. and N.G.F.M.W.; Software, R.A.I. and N.A.H.; Resources; R.A.I. and N.A.H.; Supervision, G.F.M.G., R.A.I. and N.G.F.M.W.; Visualization, M.R.B., R.A.I. and N.G.F.M.W.; Validation, M.R.B., R.A.I. and N.G.F.M.W.

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