Aminoglycoside Susceptibility among Extensive Drug Resistant *Pseudomonas aeruginosa* from Hospitalized Patients in Al-Diwaniyah, Iraq

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Abstract

Background: *Pseudomonas aeruginosa* is one of the most prevalent Gram-negative microbes associated with serious and fatal nosocomial infections. It is routine to use aminoglycosides as a treatment for *P. aeruginosa* infections. **Objectives:** The dissemination of aminoglycoside resistance offers a major challenge to the treatment of life-threatening infections caused by these bacteria. **Aim of Study:** Determination of aminoglycoside-modifying enzymes and 16S ribosomal RNA methylases in extensive-drug resistant (XDR) *P. aeruginosa* isolates. **Materials and Methods:** From November 2021 to August 2022, a total of 200 samples were collected in this cross-sectional study including burns (n = 80, 40%), wounds (n = 66, 33%), and diabetic foot ulcers (n = 54, 27%) from admitted patients to Al-Diwaniyah Burn Center and Al-Diwaniyah Teaching Hospital. The identification and antibiotic susceptibility profile of *P. aeruginosa* were done by vitek2 compact system. The isolates were subjected to polymerase chain reaction assays with specific primers for *ant(4')-IIa*, *ant(4')-IIb*, *acc(6')-Ia*, *aph(3')-IIb*, *rmtA*, and *rmtD*. **Results:** The recovery rate of *P. aeruginosa* isolates was (n = 50, 25.0%) from the clinical samples. Antibiotic-susceptibility patterns demonstrated that 18% of the isolates were multi-drug resistant and 22 (44%) were XDR. The XDR isolates were resistant to all 14 antibiotics related to the seven antibiotic classes tested in this study. The prevalence of aminoglycoside resistance genes among XDR isolates is *ant(4')-IIa* (22.7%), *(4')-IIb* (27.3%), *acc(6')-Ia* (18.18%), *aph(3')-IIb* (100%), *rmtA* (36.36%), *rmtD* (36.36%), *mexZ and parR* (100%). **Conclusion:** Increased resistance to aminoglycosides in Al-Diwaniyah Hospitals serves to highlight how critical this issue is when treating multidrug-resistant *P. aeruginosa* infections that are life-threatening. All the resistance isolates harbored *aph(3')-IIb* gene.

Keywords: 16S ribosomal RNA methylases, aminoglycoside resistance, aminoglycoside-modifying enzymes, XDR *P. aeruginosa*

Introduction

Pseudomonas aeruginosa is an opportunistic bacterium associated with a high rate of mortality and morbidity in immune-compromised patients,[1] where it causes a wide variety of acute and chronic life-threatening illnesses. Anti-pseudomonal antibiotic regimens, such as those used to treat endocarditis, bacteremia, and pulmonary infections in people with bronchiectasis and cystic fibrosis,[2,3] frequently include aminoglycosides such as amikacin, tobramycin, and gentamicin.

In healthcare settings, aminoglycosides play a significant role as broad-spectrum antibiotics. They are employed in the treatment of serious and potentially fatal hospital-acquired infections that originate from Gram-negative bacteria.[4]

Aminoglycosides that have a 2-deoxystreptamine core bind preferentially to helix 44 of the bacterial 30S ribosomal subunit-containing 16S ribosomal RNA (rRNA). Protein synthesis is disrupted by aminoglycoside binding in a

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variety of ways, including by impairing transfer RNA translocation, reducing translational fidelity, interfering with the mobility of ribosome subunits, impairing ribosome recycling, and impairing the creation of intersubunit bridges.[5,6] Additionally, aminoglycosides bind to helix 69 of the 23S rRNA in 50S ribosomal subunits and may prevent protein synthesis there.[6-8]

In Gram-negative bacteria, the aminoglycosides resistance mechanisms mainly result from: The aminoglycoside-modifying enzymes (AMEs) production, inactivating enzymes production of numerous families which are aminoglycoside nucleotidyl transferases (ANTs), aminoglycoside acetyltransferases (AACs), and aminoglycoside phosphoryl transferases (APHs); 16S rRNA methylation by ribosomal methyltransferase enzymes family; 30S ribosomal subunit mutation; antibiotics are actively expelled from bacterial cells by efflux pumps, and the permeability of cell membranes is altered along with the intracellular concentration of aminoglycosides.[9,10] Subclasses of AMEs can be differentiated according to modification site and the resistance spectrum within the category of antimicrobials.

Clinical isolates of both Gram-negative and Gram-positive bacteria have been shown to produce AMEs, which are responsible for enzymatically modifying the hydroxyl or amino groups of the medication and preventing it from binding to ribosomes and therefore allowing the bacteria to survive.[11,12]

Methylation of 16S rRNA is a recent strategy for Enterobacteriaceae and glucose-non fermentative Gram-negative germs, like *P. aeruginosa* and *Acinetobacter* spp., to counter aminoglycosides action. This mechanism is coordinated by a recently discovered family of enzymes called 16S rRNA methylases. Resistance to all currently used aminoglycosides in the clinic is greatly increased in their presence. The genes for this novel resistance mechanism are typically located on transposons within transferrable plasmids, allowing for their potential horizontal spread. This may help to explain why it has already spread so widely across the globe.[13]

However, in 2003, it was revealed that several strains of clinical *P. aeruginosa* and *Klebsiella pneumoniae* generated 16S rRNA methylases. Amikacin, tobramycin, and gentamicin are all clinically relevant aminoglycosides; however, these enzymes were discovered to induce extremely high resistance levels to them.[14]

Materials and Methods

In total, 200 clinical samples were taken from patients distributed as 80 burns, 66 wounds, and 54 diabetic foots. The patients were hospitalized at Al-Diwaniyah Teaching Hospital and Al-Diwaniyah Burn Center during the period from November 2021 to August 2022. *Pseudomonas aeruginosa* was isolated from 25 burns, 15 wound, and 10 diabetic foot clinical samples.

Under safety handling conditions, the clinical samples were obtained from patients with sterile swabs of transport media and instructed with patients' information, then transported to the laboratory. All samples were streaked based on standard procedures by using differential and selective media (MacConkey, blood, chromogenic agar) for the detection of *P. aeruginosa* and incubated at 37ºC for 24 h aerobically.[15] Confirmative diagnosis of isolates was achieved using conventional biochemical tests and confirmed by the Viteck2 compact system (Biomerieux, France, Card type: GN, ID-N222).

Pseudomonas aeruginosa isolates were subjected to antibiotics susceptibility test by Vitek2 compact system (Biomerieux, Card type: GN, AST-N222). All isolates were examined against 14 antibiotic agents related to seven antibiotic classes. According to Clinical and Laboratory Standards Institute 2021[16] recommendations, all results were interpreted and all *P. aeruginosa* isolates were classed as susceptible, intermediate, or resistant to each tested antibiotics agent.

Genomic DNA was extracted from *P. aeruginosa* isolates according to instructions of the manufacturer Genomic DNA purification kit (Geneaid). The purity and concentration of DNA for each isolate were measured by the Nanodrop instrument (THERMO, USA).

Polymerase chain reaction (PCR) was employed for screening the aminoglycosides modifying enzyme genes: *ant(4')-IIa*, *ant(4')-IIb*, *acc(6')-Ia*, *aph(3')-IIb*, and 16rRNA methylase genes (*rmtA* and *rmtD*). In this study, all primers were provided by the Macrogene company, Korea. Primers details are tabulated in Table 1.

Ethical approval

The study was carried out following the ethical principles that have their origin in the Declaration of Helsinki. Before sampling, the approval of the patient or his companion was taken. The study protocol and the subject information and the consent form were reviewed and approved by the College of Medicine, Al-Qadisiyah University according to document number 30/3666 on November 9, 2021 to get this approval.

Results

Out of 200 collected samples only 136 (68%) samples gave positive results for culturing, and out of 136 positive culturing samples, only 50 isolates (25%) were identified to be *P. aeroginosa* depending on culture characteristics and biochemical tests [Table 2].

Under the recommendations of the Clinical and Laboratory Standards Institute 2021,[16] all results were

interpreted and all *P. aeruginosa* isolates were classified as susceptible, intermediate, or resistant to each tested antibiotics agent [Table 3].

According to a study, isolates were defined as multi-drug resistant (MDR), and extensive-drug resistant (XDR) to characterize patterns of multiple drug resistance. In line

Table 1: Aminoglycoside resistance genes of *Pseudomonas aeruginosa*

N	Gene		Primer sequence	Amplicon size(bp)	Annealing temp./time	Reference
1	*ant(4')-IIa*	F	ATGCACCTCACCATTACCTACTG	759	56ºC/30 s	[17]
		R	TCACGTTCTGGCCGATATACGC			
2	*ant(4')-IIb*	F	TAT CTC GGC GGT CGA GT	364	60ºC/30 s	[18]
		R	CAC GCG GGG AAA CGC GAG AA			
3	*acc(6')-Ia*	F	GAATATTGCGGAATGCAGC	487	56ºC/30 s	[17]
		R	GGCATTTGGAATTATTCC			
4	*aph(3')-IIb*	F	ATGCATGATGCAGCCACCTCCAT	813	56ºC/30 s	[19]
		R	CCTACTCTAGAAGAACTCGTCCA			
5	*rmtA*	F	CTA GCG TCC ATC CTT TCC TC	635	55ºC/30 s	[18]
		R	TTG CTT CCA TGC CCT TGC C			
6	*rmtD*	F	CGG CAC GCG ATT GGG AAG C	401	55ºC/30 s	[13]
		R	CGG AAA CGA TGC GAC GAT			
7	*mexZ*	F	TATGATCTGCGGCGCCTTTC	883	56ºC/30 s	[20]
		R	TTCGGAACAAGGCGTCTGCA			
8	*parR*	F	ATCTCGAACGAGTCGCTGGAG	881	56ºC/30 s	[20]
		R	GTAGAACGCGTCGATGACATGG			

Table 2: Distribution of *P. aeruginosa* isolates according to the source of samples

Sample source	Total number	Bacterial growth		
		No growth	Gram- positive and negative isolates	Suspected isolates of *P. aeruginosa*
Burns	80	22 (27.5%)	58 (72.5%)	25 (43.1%)
Wounds	66	26 (39.4%)	40 (60.6%)	15 (37.5%)
Diabetic foot ulcers	54	16 (29.6%)	38 (70.4%)	10 (26.3%)
Total	200	64 (32%)	136 (68%)	50 (36.8%)
$\chi 2$			2.54	2.79
P value			0.281	0.247

* No significant difference at $P < 0.05$

Table 3: Antibiotics susceptibility patterns of *Pseudomonas aeruginosa* isolates

Antibiotics classes	Antibiotics	Sensitive (S)		Intermediate (I)		Resistant (R)	
		Isolates No.	%	Isolates No.	%	Isolates No.	%
Cephalosporins	Cefazolin (3rd G)	21	42%	0	0%	29	58%
	Ceftazidime (3rd G)	18	36%	4	8%	28	56%
	Cefepime (4th G)	25	50%	1	% 2	24	48%
Carbapenem	Imipenem	28	56%	0	0%	22	40%
	Meropenem	28	56%	1	4%	21	42%
Penicillin	Augmentin	11	22%	3	6%	36	72%
	Piperacillin	8	16%	0	0%	42	84%
Quinolone	Ciprofloxacin	21	42%	0	0%	29	58%
	Levofloxacin	26	52%	0	0%	24	48%
Aminoglycoside	Gentamicin	18	36%	0	0%	32	64%
	Tobramycin	20	40%	0	0%	30	60%
	Amikacin	22	44%	0	0%	28	56%
Macrolide	Azithromycin	24	48%	0	0%	26	52%
Glycylcycline	Tigecycline	22	44%	0	0%	28	56%
χ^2				68.004*			
P value				0			

* Significant difference at $P < 0.05$

with the definition of MDR *P. aeruginosa*, 9/50 (18%) isolates were found to be MDR [Table 4]. Furthermore, 22/50 (44%) isolates were recognized as XDR "nonsusceptible isolates to at least one agent in all but two or fewer anti-pseudomonal antibiotic categories.[21]" More seriously, 21/50 (42.0%) of them were resistant to all 14 antibiotics belonging to seven categories tested in the current study.

The resistance rate of all 22 XDR *P. aeruginosa* isolates against aminoglycoside agents: amikacin, gentamicin, and tobramycin were 100%.

Subsequently, all of the 22 XDR *P. aeruginosa* isolates were evaluated for the presence of selected aminoglycoside resistance genes including Aminoglycoside modifying enzymes: ANTs, *ant(4')-IIa*, *ant(4')-IIb*, AAC, *acc(6')-Ia* and APHs, *aph(3')-IIb*, plasmid-mediated 16S rRNA methylase genes: *rmtA* and *rmtD*, and aminoglycoside Resistance-Nodulation-Division (RND) efflux pump regulatory genes: *mexZ* and *parR* [Figures 1-8]. The distribution rate of aminoglycoside resistance genes in 22 XDR *Pseudomonas aeruginosa* isolates is shown in Table 5.

Table 4: The distribution of antibiotics susceptibility patterns of 50 *Pseudomonas aeruginosa* isolates from different clinical sources

Clinical sample	Isolate No.	Antibiotics susceptibility patterns		
		Susceptible	MDR	XDR
Burn	25	8 (40%)	6 (24%)	11 (44%)
Wound	15	7 (46.7%)	2 (13.3)	6 (40%)
Diabetic foot ulcer	10	4 (40%)	1 (10%)	5 (50%)
Total	50	19 (38%)	9 (18%)	22 (44%)
χ^2			1.71*	
P value			0.788	

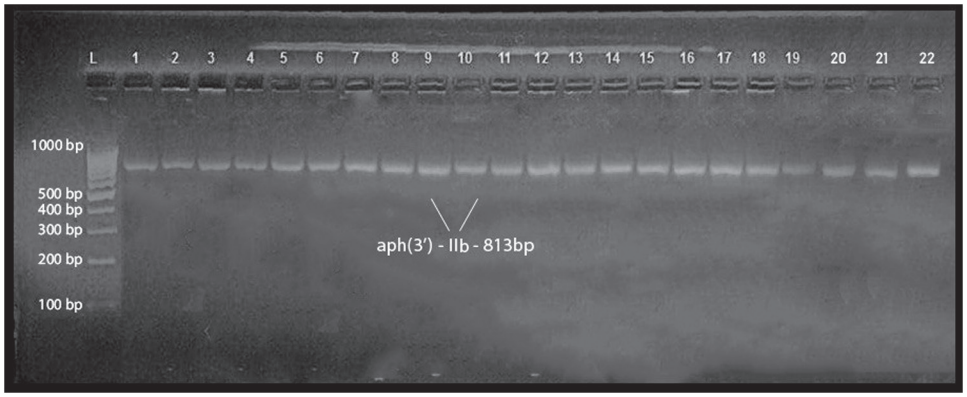

Figure 1: Agarose gel electrophoresis (1.5%) of PCR amplified products of aminoglycosides resistance *aph(3')-IIb* gene for 1 h at 70 V. Lane L: DNA marker (1500–100) bp. Lanes 1–22: Extensive multidrug resistance *Pseudomonas aeruginosa* isolates that showed positive *aph(3')-IIb* gene at 813 bp PCR product size

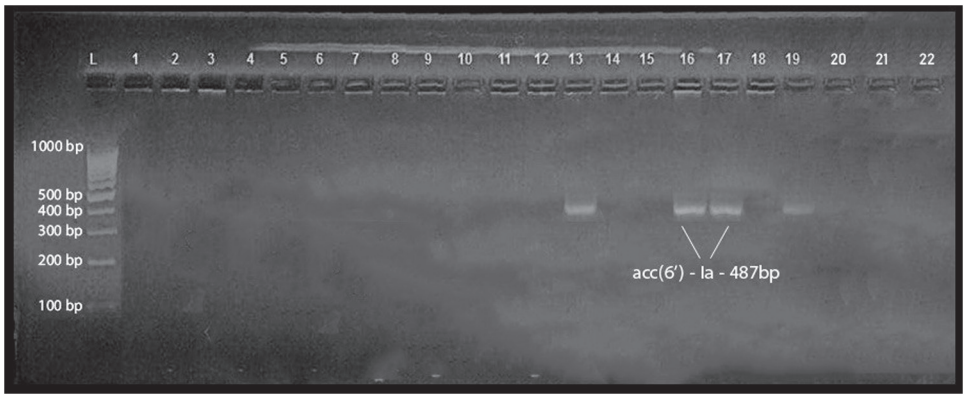

Figure 2: Agarose gel electrophoresis (1.5%) of PCR amplified products of aminoglycosides resistance *acc(6')-Ia* gene for 1 h at 70 V. Lane L: DNA marker (1500–100) bp. Lanes 1–22: Extensive multidrug resistance *Pseudomonas aeruginosa* isolates that showed positive *acc(6')-Ia* gene at 487 bp PCR product size

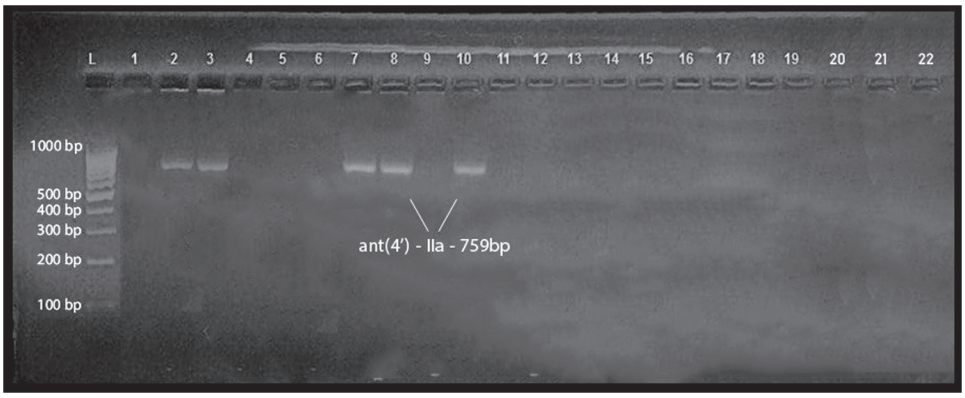

Figure 3: Agarose gel electrophoresis (1.5%) of PCR amplified products of aminoglycosides resistance *ant(4')-IIa* gene for 1 h at 70 V. Lane L: DNA marker (1500–100) bp. Lanes 1–22: Extensive multidrug resistance *Pseudomonas aeruginosa* isolates that showed positive *ant(4')-IIa* gene at 759 bp PCR product size

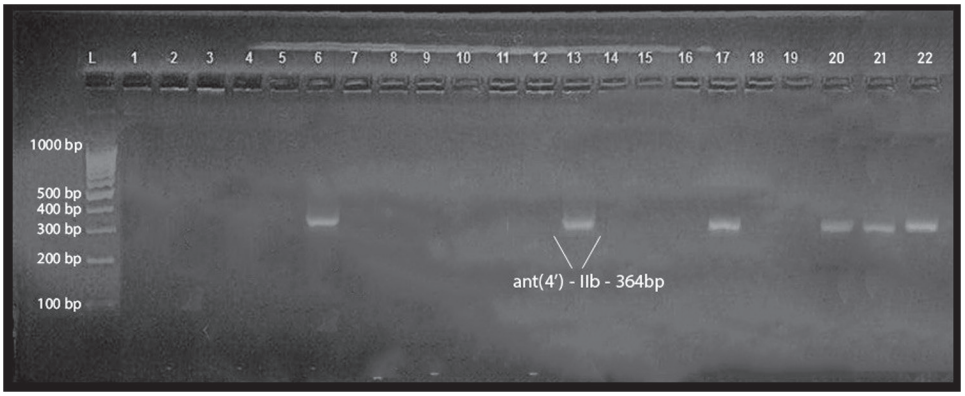

Figure 4: Agarose gel electrophoresis (1.5%) of PCR amplified products of aminoglycosides resistance *ant(4')-IIb* gene for 1 h at 70 V. Lane L: DNA marker (1500–100) bp. Lanes 1–22: Extensive multidrug resistance *Pseudomonas aeruginosa* isolates that showed positive *ant(4')-IIb* gene at 364 bp PCR product size

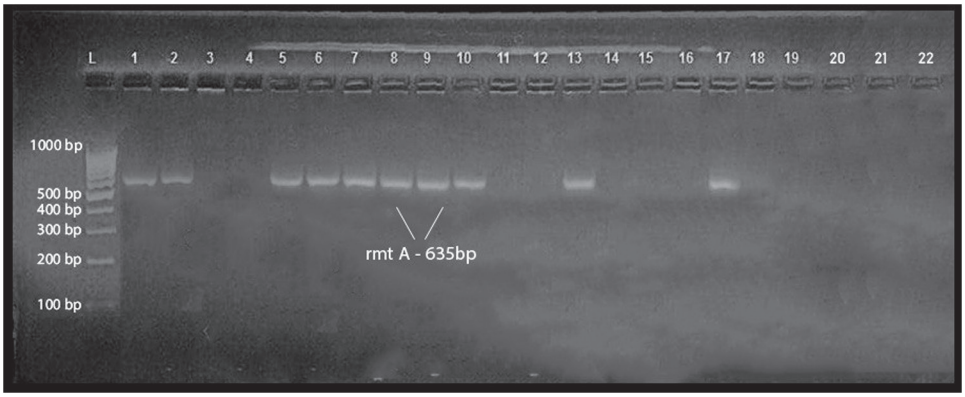

Figure 5: Agarose gel electrophoresis (1.5%) of PCR amplified products of aminoglycosides resistance *rmtA* gene for 1 h at 70 V. Lane L: DNA marker (1500–100) bp. Lanes 1–22: Extensive multidrug resistance *Pseudomonas aeruginosa* isolates that showed positive *rmtA* gene at 635 bp PCR product size

Discussion

Multidrug resistance frequently arises from the acquisition of external resistance genes and/or through mutational-associated resistance.[22] Multiple antibiotic-resistant isolates are developed as a result of the diversity of antibiotic resistance mechanisms which makes conventional antibiotics ineffective for the treatment of *P. aeruginosa* infections.[23]

Compared to the data obtained on extended spectrum beta-lactamase-producing and carbapenem-resistant

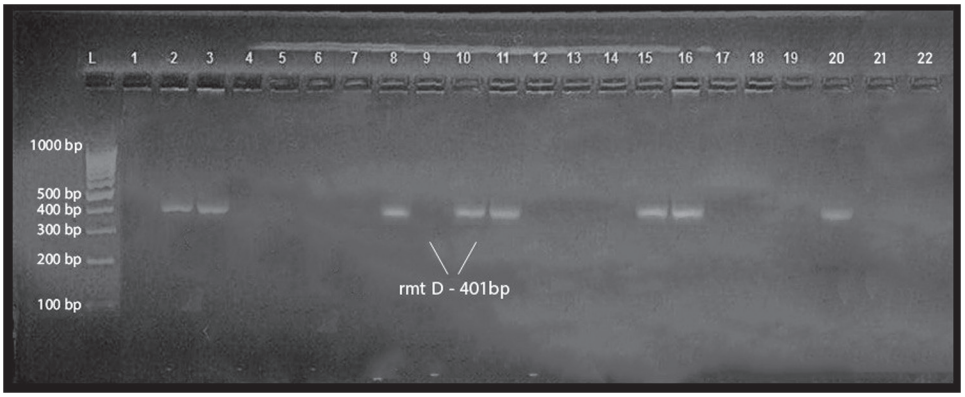

Figure 6: Agarose gel electrophoresis (1.5%) of PCR amplified products of aminoglycosides resistance *rmtD* gene for 1 h at 70 V. Lane L: DNA marker (1500–100) bp. Lanes 1–22: Extensive multidrug resistance *Pseudomonas aeruginosa* isolates that showed positive *rmtD* gene at 401 bp PCR product size

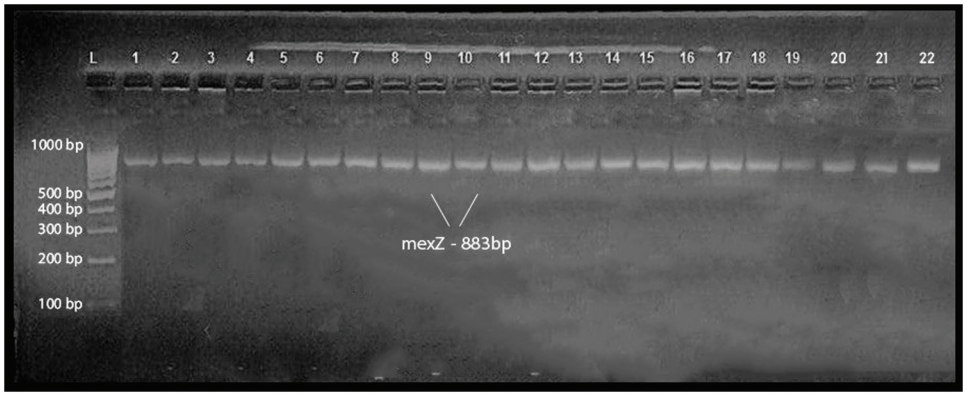

Figure 7: Agarose gel electrophoresis (1.5%) of PCR amplified products of aminoglycosides resistance *mexZ* gene for 1 h at 70 V. Lane L: DNA marker (1500–100) bp. Lanes 1–22: Extensive multidrug resistance *Pseudomonas aeruginosa* isolates that showed positive *MexZ* gene at 883 bp PCR product size

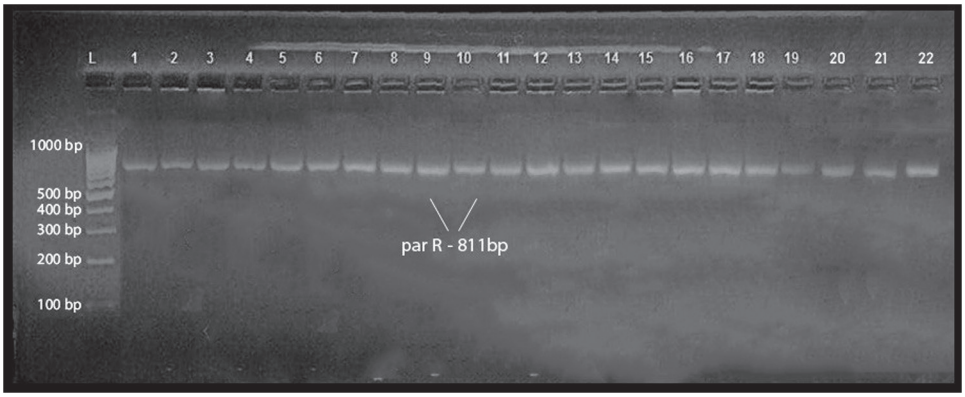

Figure 8: Agarose gel electrophoresis (1.5%) of PCR amplified products of aminoglycosides resistance *parR* gene for 1 h at 70 V. Lane L: DNA marker (1500–100) bp. Lanes 1–22: Extensive multidrug resistance *Pseudomonas aeruginosa* isolates that showed positive *parR* gene at 881 bp PCR product size

P. aeruginosa, there are few study data on the prevalence of genes confer aminoglycoside resistance in XDR *P. aeruginosa* isolates in Al-Diwaniyah hospitals.

This study documented that all XDR *P. aeruginosa* isolates exhibited *aph(3')-IIb*. Consistent with previous findings, *aph(3′)-IIb* can be found in virtually all *P. aeruginosa* isolates.[24]

In Al-Diwaniyah, studies reported similar results, recording *aac(6')-Ib* gene as the highest prevalence among the resistant isolates.[25,26]

Table 5: Distribution of aminoglycoside resistance genes among XDR *Pseudomonas aeruginosa* isolates

Isolate code No.	Aminoglycoside resistance genes							
	ant(4')-IIa	ant(4')-IIb	acc(6')-Ia	aph(3')-IIb	rmtA	rmtD	mexZ	parR
Pa1	–	–	–	+	+	–	+	+
Pa2	+	–	–	+	+	+	+	+
Pa3	+	–	–	+	–	+	+	+
Pa4	–	–	–	+	–	–	+	+
Pa5	–	–	–	+	+	–	+	+
Pa6	–	+	–	+	+	–	+	+
Pa7	+	–	–	+	+	–	+	+
Pa8	+	–	–	+	+	+	+	+
Pa9	–	–	–	+	+	–	+	+
Pa10	+	–	–	+	+	+	+	+
Pa11	–	–	–	+	–	+	+	+
Pa12	–	–	–	+	–	–	+	+
Pa13	–	+	+	+	–	–	+	+
Pa14	–	–	–	+	–	–	+	+
Pa15	–	–	–	+	–	+	+	+
Pa16	–	–	+	+	–	+	+	+
Pa17	–	+	+	+	–	–	+	+
Pa18	–	–	–	+	–	–	+	+
Pa19	–	–	+	+	–	–	+	+
Pa20	–	+	–	+	–	+	+	+
Pa21	–	+	–	+	–	–	+	+
Pa22	–	+	–	+	–	–	+	+
Percentage	22.7%	27.3%	18.18%	100%	36.36%	36.36%	100%	100%
X2				26.28*				
P value				0				

Pa: *Pseudomonas aeruginosa*

* Significant difference at $P < 0.05$

In regard to ANTs, the present study documented that XDR *P. aeruginosa* isolates harbored *ant(4')-IIa* and *ant(4')-IIb* with 22.7% and 27.3%, respectively. To our knowledge, the prevalence of *ant(4')-IIa* gene has been investigated for the first time. The *ant(4')-IIa* and *ant(4')-IIb* were found to be encoded on plasmids from Enterobacteriaceae and *P. aeruginosa*, respectively. These genes confer resistance to tobramycin, amikacin, and isepamicin but not to gentamicin.[11]

The 16S-RMTases have become a new mechanism of aminoglycoside resistance since 2003. Their plasmid-encoded genes are found in many different bacterial species, including Enterobacteriaceae and *P. aeruginosa*, and have spread around the world. All aminoglycosides given parenterally that are now used in clinical settings are resistant to an extraordinarily high level due to these genes.[27,28] This study recorded that 36.36% of XDR *P. aeruginosa* isolates harbored *rmtA* and *rmtD* genes.

Most of the Iraqi studies recorded a low to no prevalence rate for this gene among isolates,[25,29] and the percentage obtained by this study is relatively high compared to those studies. This may give an alarm bell to the possibility of starting to spread these genes in health institutions.

In Al-Najaf hospitals, the previous study recorded (13.6%) of *ant(4')-IIb* gene distributed among collected XDR isolates.[29]

The aminoglycoside resistance genes frequency in XDR *P. aeruginosa* was showed (93.75%, 87.5%, 25%, and 0.0%) for (*aac(6')-Ib*, *aph(3')-VI aac(6')-II*, and, *ant(2')-I*), respectively.[26]

High-level resistance to the effective therapeutic aminoglycosides is conferred by methylation of the 16S rRNA within the 30S ribosomal subunit. This mechanism found only in Gram-negative pathogens was first identified in 2003 and is now being reported increasingly often across the whole world. The combination of 16S rRNA methylase genes with genetic recombination systems, which is prevalent among pathogens that produce 16S rRNA methylase, frequently promotes the development of multidrug resistance, mainly versus broad spectrum lactams, via ESBLs or metallolactamases production.[13]

The most common 16S RMTase genes that modulate aminoglycoside resistance among bacterial isolates in Korean communities were ArmA and rmtB. Since armA and rmtB have been found all over the world and other

16S RMTase genes "*rmtA, rmtC, rmtD, rmtF, rmtG*, and *rmtH*" are found in different parts of the world.[30]

The spread of 16S-RMTases among resistant *P. aeruginosa* is not an ideal event. To impede the spread of these resistant isolates, strict infection control actions need to be established in practice.

Whereas *P. aeruginosa* employs various mechanisms to circumvent the effects of antibiotics, the activity of energy-dependent efflux pumps linked to the RND superfamily is largely responsible for the organism's intrinsic resistance to numerous antibiotics.[31]

The RND pumps transcriptional regulation is generally mediated by a regulatory protein encoded upstream of the RND operon.[32] But it is becoming clear that the transcriptional regulation of RND pumps in organisms like *P. aeruginosa* is quite complicated[31] and that RND pump overexpression can happen not only in reaction to antimicrobials but also to different environmental stresses.[33]

High resistance rates exhibited by *P. aeruginosa* to antibiotics render treatment of its infections, particularly challenging. However, recent local studies have demonstrated how far antibiotic resistance has spread among pathogenic bacterial isolates through several resistance mechanisms and distinct resistance patterns.[34-36] Because this raises concern and requires urgent intervention.

This requires aspiration to find solutions and extensive local studies to find alternative solutions to antibiotics or to combine them with them. Recently, local studies dealt with this aspect and proved the effectiveness of alternative methods for treating resistant bacteria.[26,37,38]

Conclusion

Increased aminoglycoside resistance in Al-Diwaniyah Hospitals highlights the importance of this problem while managing life-threatening multidrug-resistant *P. aeruginosa* infections.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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