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Molecular Analysis of Carbapenem Resistant Genes in *Pseudomonas aeruginosa* isolated from Baghdad Hospitals

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Abstract

Objectives: This study aimed to molecular investigation of prevalence the carbapenem-resistant genes in *P. aeruginosa* in isolates collected from Baghdad hospitals.

Methods: In a cross-sectional manner, *P. aeruginosa* were isolated and identified from patients who attended to Hospital in Baghdad city during the period of December 2021 to June 2022. Genotypic characterization of *16SrRNA* gene, plasmid profile, *exoA* gene, carbapenem resistance gene were tested.

Results: Diagnosis of *P. aeruginosa* isolates was confirmed genotypically via the amplification of *16SrRNA* gene with 1504 bp by using PCR amplification of genes encoding Ambler class B MBL (blaIMP, blaVIM, *IMI*, *bla*NDM, *bla*SPM-1, and *bla*GIM), and Ambler class D carbapenemase (*blaOXA-23*, *blaOXA-24,* *bla*OXA-40, *bla*OXA-48, *bla*OXA-50, *blaOXA-51* and *blaOXA-58*) were performed. The carbapenem resistant isolates were also evaluated for the presence of class D carbapenemase (*blaOXA 50*), genes by PCR.

Conclusion: This study proved that *P. aeruginosa* isolated had carbapenem-resistant genes that strongly correlated with antibiotic resistance according to phenotypic and genotypic characterization.

Keywords: Carbapenems, *Pseudomonas aeruginosa*, Hospitals, Iraq

Introduction

Pseudomonas aeruginosa is a ubiquitous, Gram-negative bacterium and versatile opportunistic pathogen, which is considered a significant reason for an ever-widening array of various life-threatening infections.[1] Over the past decades, the emergence and dissemination of *P. aeruginosa* and Enterobacteriaceae, which are resistant to carbapenems, which are the broadest spectrum agents of the β-lactam group, has become apparent as an urgent threat to public health.

The finding *P. aeruginosa* has intrinsic resistance to numerous antimicrobial agents and also easily acquires resistance to many antibiotics, including carbapenems resistance is an ominous development that challenges this last-resort antibiotic. Unfortunately, carbapenems resistant *P. aeruginosa* has now emerged and is disseminating worldwide.[2-3] Resistance to carbapenems in *P. aeruginosa* can be mediated by several mechanisms, including MexAB-OprM, AmpC, decreased outer membrane permeability, up-regulation of the efflux pumps, hyper production of a chromosomal AmpC-type cephalosporinases or the production of carbapenemases OprD.[4]

There is scarce information available on the distribution of MBLs producing *P. aeruginosa* isolates in Baghdad hospitals. Therefore, immediate determination of carbapenemases and other mechanisms creating isolates is main to avoid the spread of *P. aeruginosa* inside and between hospitals and to correctly treat infections caused by this. The study aimed to identify the variations in phenotypic, genotypic characteristics and antibiotic resistance profile of *Pseudomonas aeruginosa* isolated from inpatients in Baghdad hospitals. Additionally, the use of carbapenems has increased markedly during the past few years in Baghdad city. The current study suspected that increased use of these antibiotics could cause the selection of isolates resistance to carbapenems.

Materials and Methods

Isolation and Identification of P. aeruginosa

A total of 2000 clinical samples were collected from inpatients of many hospitals in Baghdad city during the period from December 2020 to June 2021, which included: diabetic foot (50), otitis media (550), lower respiratory tract (150), urinary tract (725), wound (200), Blood and burns (250). The clinical samples were transported to the laboratory without delay. All samples were cultivated, by using the standard loop of urine and sterile swabs of other samples, on the blood agar, MacConkeys agar Pseudomonas chromogenic agar, and cetrimide agar as selective media. *P. aeruginosa* and incubated overnight at 37°C for 18–24 hours. Initial diagnosis of isolates was made on the basis of Gram's staining of culture, colonial morphology on different media, hemolysis on blood agar, pigment production, odor in cultures, size, edge, and oxidase test. Suspected Pseudomonas colonies were further identified to species level using routine biochemical tests and selective culture media.[5] In addition to these tests, the *P. aeruginosa* isolates were also confirmed biochemically with the Vitek-2 automated system and by *16SrRNA* as a molecular method.

Oligonucleotide Primer Sequences Used for PCR Amplification

1. Specific primer sequences of *16SrRNA* gene were used to confirm the identification of *P. aeruginosa* by PCR according to Jiang et al., 2006[6] provided by Alpha DNA company (Canada) and prepared according to the instructions of the supplied company, as shown in Table 1.
2. Oligonucleotide primer for detection of carbapenem resistance genes in *P. aeruginosa*. These primers were provided by Macrogen company from South Korea are listed in Table 2.

Table 1. **Oligonucleotide primer sequences of *16SrRNA* gene in *P. aeruginosa* used for confirmatory identification**

Name of primer	Primer sequences (5′→3′)		Reference	Product size
16SrRNA	F	AGA GTT TGA TCM TGG CTC AG	(Jiang et al., 2006)[6]	bp1504
	R	CGG TTA CCT TGT TAC GAC TT		

Table 2. **Oligonucleotide primer for detection of carbapenem resistance genes in *P. aeruginosa***

Primer	Gene name	Sequence (5′→3′)		Product size	Genbank reference
NDM	*bla-NDM*	F	CAGTCGCTTCCAACGGTTTG	529 bp	MF379690.1
		R	ATCACGATCATGCTGGCCTT		
IMP	*bla-IMP*	F	CTTTCAGGCAGCCAAACCAC	371 bp	Design to this study
		R	TGGGGCGTTGTTCCTAAACA		
VIM-1	*bla-VIM-1*	F	TCCACGCACTTTCATGACGA	503 bp	Design to this study
		R	AAGTCCCGCTCCAACGATTT		
GIM	*blaGIM*	F	AGAACCTTGACCGAACGCAG	909 bp	Design to this study
		R	GCACCAGTTTTCCCATACAG		
OXA-48	*blaOXA-48*	F	TTG GTG GCA TCG ATT ATC GG	744 bp	Design to this study
		R	GAG CAC TTC TTT TGT GAT GGC		
OXA-40	*blaOXA-40*	F	CACCTATGGTAATGCTCTTGC	491 bp	(Woodford et al., 2006)[7]
		R	GTGGAGTAACACCCATTCC		
OXA-50	*blaOXA-50*	F	AATCCGGCGCTCATCCATC	869 bp	(Woodford et al., 2006)[7]
		R	GGTCGGCGACTGAGGCGG		
SPM	*blaSPM-1*	F	CCTACAATCTAACGGCGACC	650	(Woodford et al., 2006)[7]
		R	TCGCCGTGTCCAGGTATAAC		
Oxa58	*bla Oxa58*	F	AAGTATTGGGGCTTGTGCTG	599	(Woodford et al., 2006)[7]
		R	CCCCTCTGCGCTCTACATAC		
Oxa23	*bla Oxa23*	F	GATCGGATTGGAGAACCAGA	501	(Woodford et al., 2006)[7]
		R	ATTTCTGACCGCATTTCCAT		
Oxa24	*bla Oxa24*	F	GGTTAGTTGGCCCCCTTAAA	246	(Woodford et al., 2006)[7]
		R	AGTTGAGCGAAAAGGGGATT		
Oxa 51	*blaOxa 51*	F	TAATGCTTTGATCGGCCTTG	353	(Woodford et al., 2006)[7]
		R	TGGATTGCACTTCATCTTGG		
IMI	*blaIMI*	F	CCA TTC ACC CAT CAC AAC	440	(Woodford et al., 2006)[7]
		R	CTA CCG CAT AAT CAT TTG C		

PCR Protocols for Detection of Carbapenem Resistance Genes

PCR protocols for detection of carbapenem resistance genes. An oligonucleotide primer was prepared depending on the manufacturer's instruction by dissolving the lyophilized sample with nuclease-free water after rotating down briefly. A working primer tube was prepared by diluting it with nuclease-free water. The final pico-moles depended on the procedure of each primer. The PCR tubes were placed into a thermocycler and the right PCR cycling program parameters conditions were installed as in Table 3. PCR was used for detecting *P. aeruginosa*, the mixture of 25 µl consisted of 12.5 µl of GoTaq Hot Star master mix (which contains Taq DNA polymerase, dNTPs, $mgcl_2$, and reaction buffer at the optimal concentration for efficient amplification of DNA templates by PCR), 5 µl DNA template (20 ng.), 1 µl of each forward and reverse primer (10 pmol.) 5.5 µl of nuclease-free water to complete the amplification mixture volume. The PCR tubes containing the mixture were transferred to preheated Thermo cycler under sterile condition.

All requests, technical and preparations of agarose gel electrophoresis that were used for the detection of PCR products were done according to Sambrook and Russel.[8] The PCR products separated in 1.5% agarose gels (after staining with 0.5 mg/ml Ethidium bromide) were visualized using a gel ultraviolet transilluminator system. The positive results were distinguished when the PCR product base pairs were equal to the base pairs of the DNA ladder.

Results and Discussion

According to the results of the present study, the overall count constitutes a total of 100/2000 samples, *P. aeruginosa*

Table 3. **Programs of PCR thermocycling conditions for detection of carbapenem resistance genes**

Genes	Temperature (C°)/Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		Denaturation	Annealing	Extension		
NDM	95°C/5 min	95°C/20 sec	56°C/30 sec	72°C/40 sec	72°C/5 min	35
IMP	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
VIM-1	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
GIM	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
SPM	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
OXA-48	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
OXA-40	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
OXA-50	95°C/5 min	95°C/20 sec	58°C/30 sec	72°C/40 sec	72°C/5 min	35
OXA-51	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
OXA-23	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
OXA-24	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
SPM	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35
IMI	95°C/5 min	95°C/20 sec	50–60°C/30 sec	72–40°C/40 sec	72°C/5 min	35

representing (5%) of all the collected samples in this study. It's well known that *P. aeruginosa* considers an important nosocomial pathogen in many medical centers throughout the world and a source of infections in any part of the body. Also, this bacterium is able to cause infection in healthy individuals at a low rate and creates a serious public health disaster resulting in an enormous burden of morbidity, and mortality in both developing and developed countries.[9] One of the reasons for the high pathogenicity of *P. aeruginosa* is the intrinsic high resistance to several antibiotics, as well as the development of multiple drug resistance.[10]

The results of amplification of *16SrRNA* gene with DNA extracted from one hundred *P. aeruginosa* isolates showed positive PCR product with amplicon size 1504 base pair (Figure 1). These results agreed with Alornaaouti[11] who used the *16SrRNA* gene which is considered one of the important gene to confirm the identification of *P. aeruginosa* and other bacterial species because have hyper constant sequencing and play a basic role in molecular identification and classification, also that can provide species-specific signature sequences useful for bacterial identification all type of bacteria.[12]

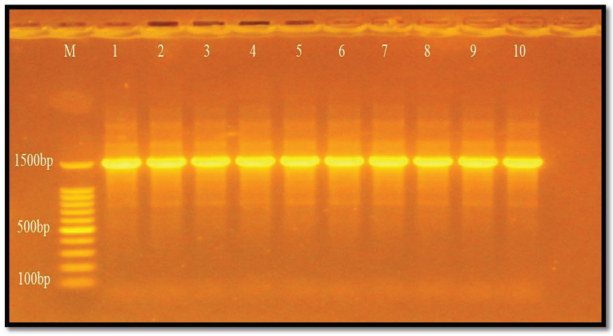

Fig. 1 **Agarose gel electrophoresis for *16SrRNA* gene amplicons. 1% agarose (100 min at 100 volt/50 mAmp). Lane:1 (M 100 bp ladder) Lane 1-10 local isolates, PCR product 1504 bp size.**

Detection of Class B (MBL) and Class D Carbapenemase

The production of carbapenemases is of the utmost concern and became the mechanism of greater relevance towards carbapenem resistance due to the growing enzyme diversity. These enzymes have high versatility, as they are characterized by a very wide hydrolytic spectrum and affect almost all β-lactams, with the exception of monobactams.[13] *P. aeruginosa* isolates producing carbapenemases are also associated with XDR phenotype. Accordingly, the detection of carbapenemases production in *P. aeruginosa* is important not only for the adequate selection of antibiotic therapy but also for hospital epidemiology surveillance and infection control.

In recent reports in Najaf, the most common carbapenemases are the MBL and OXA variant enzymes of Ambler class D.[14-16] Class B acquired MBL is one of the most important enzymes including IMP, VIM, SPM, NDM, SIM and GIM, which expose huge-level of resistance against carbapenem.[17–19]

In this study, the PCR technique was used to identify the bla-IMP, bla-VIM, bla SPM, bla-NDM, bla-SIM and bla-GIM genes only (Figure 2), while the bla-AIM, bla-KHM, bla-DIM and bla-FIM genes was not included due to very few occurrences in adjacent nations to Iraq.

Furthermore, as the present results revealed, two isolates carry the *NDM* encoding gene in addition to coexisting with the *OXA-50* encoding gene. The co-harboring of two carbapenemase genes in *P. aeruginosa* isolates has been reported in several studies worldwide.[19–21]

This observation led to the emergence of a new drug-resistant model for *P. aeruginosa*. However, this result is of great concern and has shed light on the fact that *NDM*-producing XDR *P. aeruginosa* is now alarmingly on the increase in Baghdad hospitals.

Although only three carbapenem-resistant *P. aeruginosa* isolates were identified to carry this gene in the present study, it is of concern as *blaNDM* producers may be disseminated rapidly in Baghdad hospitals and this finding implies that

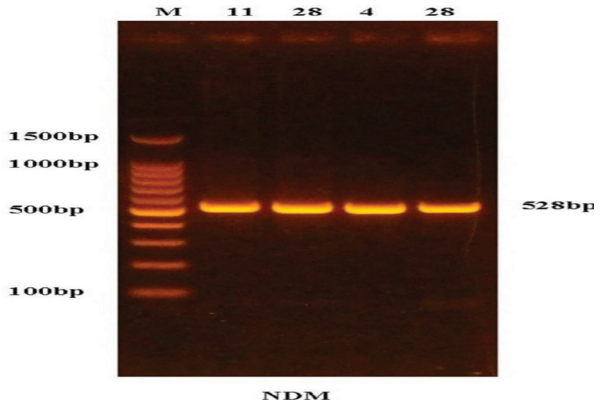

Fig. 2 **Agarose gel electrophoresis for *NDM* gene amplicons. (1.5% agarose,100 min at 100 volt/50 mAmp). Lane:1 (M 1500 bp ladder) Lane 4,11 and 28 local isolates, Positive PCR product 528 base pair.**

several new *blaNDM* cases will be found in the near future. Consequently, the detection of five *NDM*-positive isolates in this study suggested possibilities of spread via its high rate of genetic transfer among pathogenic bacteria in Baghdad hospitals, or possibilities to human factors such as hygiene and international tourists. It is believed that the emergence of *blaNDM* carried isolates in Baghdad may have been a result of the introduction of *NDM* isolates via increasing medical tourism of Iraqi patients to the Indian subcontinent.

At present, the prevalence of *NDM*-1 has increased significantly throughout the world and has been identified mostly in Asia,[19] Europe, Africa.[20] Therefore, the detection of *NDM* harboring *P. aeruginosa* isolates in this study indicates the immediate importance of the establishment of surveillance to prevent nosocomial infections and dissemination of *NDM* in Baghdad hospitals.[21-24] In the study of these genes blaSPM, IMP, VIM1, *GIM, IMI, OXA-48, OXA-40, OXA-58*, *OXA-23*, *OXA-24*, and *OXA51* were negative results (Figure 3).

In further studies conducted in Najaf, 14 showed that two (5.9%) out of 34 carbapenem-resistant *P. aeruginosa* isolates were *SPM*-type positive. Interestingly, the present study showed that *OXA-50* was the most frequent carbapenemase identified in XDR *P. aeruginosa* isolates (98.%). However, the occurring Class-D oxacillinase *OXA-50* was shown to be expressed constitutively in *P. aeruginosa* (Figure 4).

A similar enhancement in the prevalence of *blaOXA-50* carrying *P. aeruginosa* isolates has been noticed earlier in a recent local study reported by Rasool et al.,[15] who found that the majority of the carbapenems resistant *P. aeruginosa* isolates carried the *blaOXA-50* gene (53.8%). While Al-Janahi[14] reported that only 8.8% of the carbapenem resistant *P. aeruginosa* isolated from Najaf hospitals were harbored *blaOXA-50*.

Ethics Consideration

This study is in accordance with the ethics committee of Al-Diwaniaya teaching hospital, Iraq. A verbal agreement was obtained from participants in the study of the relative's pre-taking samples.

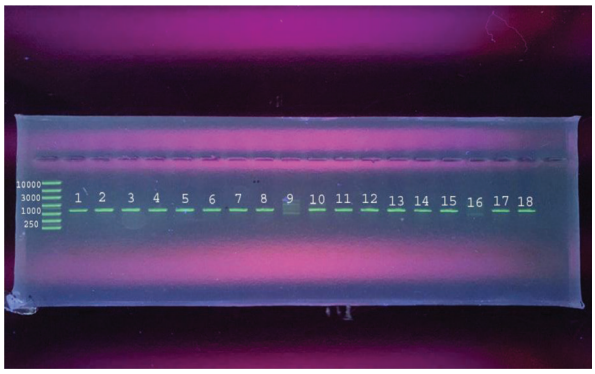

Fig. 3 **Agarose gel electrophoresis for *OXA-50* gene amplicons. (1.5% agarose (100 min at 100 volt/50 mAmp). Lane:1 (M 1500 bp ladder) Lane 1,2,3,4,5,6,7,8,10,11,12,13,14,15,17,18. Positive PCR product 869 base pair, and 2 local isolates (9,16) Negative *OXA-50* gene.**

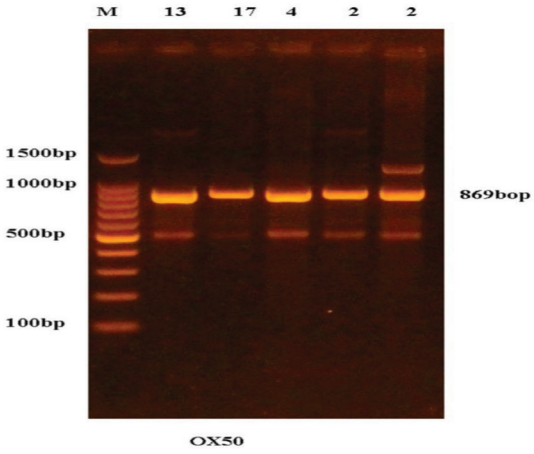

Fig. 4 **Agarose gel electrophoresis for *OXA-50* gene amplicons. (1.5% agarose, 100 min at 100 volt/50 mAmp). Lane:1 (M 1500 bp ladder) Lane 4,17,4 and 2 local isolates, Positive PCR product 869 base pair.**

Conflict of Interest

No known conflict of interest correlated with this publication.

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Availability of Data and Materials

The data used and/ or analyzed throughout this study are available from the corresponding author on reasonable request.

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