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RESEARCH ARTICLE OPEN ACCESS

Molecular Detection of Serine Carbapenemase Genes in Carbapenem-Resistant Isolates of *Pseudomonas aeruginosa* Recovered from Patients in Al-Diwaniyah Province, Iraq

Khawlah Jabbar Hussein Al-abedi and Fiars Srhan Abd Al-Mayahi

Department of Biology, College of Science, University of AL-Qadisiyah, Al Diwaniyah, Qadisiyyah Province, Iraq.

Abstract

The present study aimed at investigating the spread of serine carbapenemase-producing isolates of *Pseudomonas aeruginosa* recovered from some hospitals and specialized health centers in Al-Diwaniyah City, Iraq. Using morphological and molecular methods, 630 clinical samples (244 burn swabs, 163 sputa, 115 urine samples, and 108 ear swabs) were tested. The current work also explored the carbapenemase production ability of the carbapenem-resistant (CR) isolates using morphological and molecular methods. A monoplex polymerase chain reaction (MPCR) technique targeting 4 different serine carbapenemase (SCMs) coding genes was followed. The study also explored the nucleotide sequences of the SCM genes. The API 20 E and Vitek 2 system showed that 100 of the isolates belonged to *P. aeruginosa*. With different severity of resistance by percentages of isolates to various antibiotics, only 24% of the isolates displayed resistance against carbapenem. The results of the SCM-related MPCR showed the occurrence of the *bla*$_{GES}$, *bla*$_{SME}$, *bla*$_{KPC}$, and *bla*$_{OXA-48}$ genes in 20 (83.3%), 19 (79.1%), 11 (45.8%), 18 (75%), respectively, of the isolates. The phylogenetic study analysis revealed high, 99%, identity with NCBI-related world strains. According to the best of our knowledge, this is the first study in Iraq that detects a group of encoding genes of serine carbapenemase in carbapenem-resistant isolates.

Keywords: PCR, phylogeny, *Pseudomonas aeruginosa*, sequencing, serine carbapenemase genes.

***Correspondence:** khawlahjabbar2019@gmail.com

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INTRODUCTION

Pseudomonas aeruginosa is one of the main opportunistic pathogens, responsible for nosocomial infection and infections in cancer patients undergoing chemotherapy, cystic fibrosis, and patients with severe burns. These bacteria are linked to high morbidity and mortality rates (Schaber *et al.*, 2007; Alikhani *et al.*, 2017; Maroui *et al.*, 2017)The emergence of *P. aeruginosa* strains with multi-drug resistance (MDR) due to their possession of huge genetic modification that can develop a large number of factors associated with resistance to different antibiotics (Bassetti *et al.*, 2018). The bacterial resistance to antibiotics is thus considered as one of the major public health problems worldwide (Mohamed *et al.*, 2018). Several antibiotics have been used to treat the infections caused by these bacteria based on the Clinical & Laboratory Standards Institute: CLSI Guidelines (CLSI) including β-lactams such as carbapenem, aminoclycosides, quinolones, and lipopeptides (Institute–CLSI, 2017).

Carbapenem is a broad-spectrum antibiotic (Mohamed *et al.*, 2018). It is also one of the most potent drugs used to treat infections caused by these MDR-*P. aeruginosa* (Mirsalehian *et al.*, 2017). However, emergence of carbapenem-resistant (CR) strains have launched a major health issue around the world (Dogonchi *et al.*, 2018).

The antibiotic resistance of *P. aeruginosa* is either a self-derived process or induced by genetic material transferring using factors such as plasmids. The resistance can be initiate by certain mechanisms such as decreased antibiotic uptake due to reduced outer-cellular-membrane permeability, increased drug pumping through efflux pump overactivity, bacterial change of the antibiotic target site, and the production of carbapenemases, a potent member of the β-lactamase group (Bassetti *et al.*, 2018). Using Ambler classification, carbabenemase enzymes are classified according to their molecular properties to A, B, and D, where A and D require a serine, namely serine carbabenemase (SCMs), in their active sites to perform reactivity, while Class B, called metallo-β-lactamases (MBLs), requires Zn^{++} to break down the β-lactam ring (Jean *et al.*, 2015; Bonomo, 2017; Khan, Maryam and Zarrilli, 2017).

The present study aimed at investigating the spread of serine carbapenemase-producing isolates of *Pseudomonas aeruginosa* recovered from some hospitals and specialized health centers in Al-Diwaniyah City, Iraq.

MATERIALS AND METHODS

Patients and sample collection

In the current work, 630 clinical samples (244 burn swabs, 163 sputa, 115 urine samples, and 108 ear swabs) were collected from patients during July to December, 2018, from different hospitals and specialized health centers in Al-Diwaniyah province, Iraq. Highly aseptic conditions were followed during the sampling. The samples were then ice-box transported to a microbiological laboratory in the University of Al-Qadisiyah, Diwaniyah City, Iraq, to perform the required tests.

Cultivation, identification, sensitivity tests

MacConky and blood agars were used to grow the bacterium. The morphological and biochemical tests, API 20 E, and Vitek 2 system were utilized to recognize the identity of the bacterium. These tests were followed methods mentioned by (MacFaddin, 2000; Benson, 2002; Dulczak and Kirk, 2005; Winn, 2006; Brown, 2009; Fritsche *et al.*, 2011). The sensitivity method used was Kirby-Bauer and was performed according to (Institute–CLSI, 2017). The antibiotic sensitivity test discs (Bioanlyse Company) used in the present work were ticarcillin-clavulanat (CTC), piperacillin-tazobactam (PTZ), ciprofloxacin (CIP), levofloxacin (LEV), cefepime (FEP), ceftazidime (CAZ), imipenem (IMP), meropenem (MEM), aztreonam (ATM), amikacin (AK), gentamicin (CN), tobramycin (TOB), netilmicin (NET), Colisten (CT), and Polymyxin B (PB).

Molecular detection

Genomic DNA extraction

The DNA was extracted using a specialized kit for genomic DNA extraction manufactured by (Geneaid, USA). The process of the extraction was performed following the kit protocol provided by the mentioned company. The DNA was scanned using a NanoDrop to evaluate the quantity and the quality of the extracted DNA. The resulted DNA was stored in a deep freezer waiting to perform the next tests.

Monoplex polymerase chain reaction

A monoplex polymerase chain reaction (MPCR) technique targeting 4 different serine carbapenemase (SCMs) coding genes was used.

Table 1. The primers of the PCR

Primer		Sequence (5→3)	Product size (bp)	Reference
OXA-48	F	GCG TGG TTA AGG ATG AAC AC	438	(Poirel *et al.*, 2011)
	R	CAT CAA GTT CAA CCC AAC CG		
GES	F	TCA CTC TGC ATA TGC GTC GG	792	(Murugan *et al.*, 2018)
	R	ACT TGA CCG ACA GAG GCA AC		
KPC	F	ATG TCA CTG TAT CGC CGT CT	893	(Bradford *et al.*, 2004)
	R	TTT TCA GAG CCT TAC TGC CC		
SME	F	ACT TTG ATG GGA GGAT TGG C	551	(Poirel *et al.*, 2011)
	R	ACG AAT TCG AGA TCA CCA G		

The primers used are mentioned in table (1).

The PCR reactional solution was prepared using Accu power PCR Pre Mix (Bioneer Company, Korea). The solution was initiated with the use of 5µl DNA template, 2µl of each primer (F or R), and 11µl PCR water to complete the total volume, 20µl. The thermocycler conditions are provided in table (2).

The PCR products were electrophoresed through a 1%-agarose gel pretreated with ethidium bromide. The PCR products were then visualized under a UV imager.

Phylogenetic analysis

The DNA sequencing was performed to determine the nucleotide sequence of the carbapenemase coding genes. The PCR products were sent out to do sequencing by Macrogen Company, South Korea. The sequencing data were analyzed using the NCBI-based BLAST website, and the MEGA v6.0 was used to construct the phylogenetic tree based on the Unweighted Pair Group Methods with Arithmetic Mean (UPGMA tree) method.

Statistical analysis

The data of the study were statistically analyzed using Statistical Package for Social Science (SPSS), 23rd edition. Chi-square test was used to confirm the differences in the data. Confidence interval was equal to 95%, Probability ≤ 0.05 (P≤0.05).

RESULTS

The results of the phenotypic examination showed that the bacterial colonies were pale in color on The MacConky agar as no lactose fermentation was induced, a grape-like odor, and β-hemolysis on the blood agar. Microscopic diagnosis of bacteria showed that the bacteria

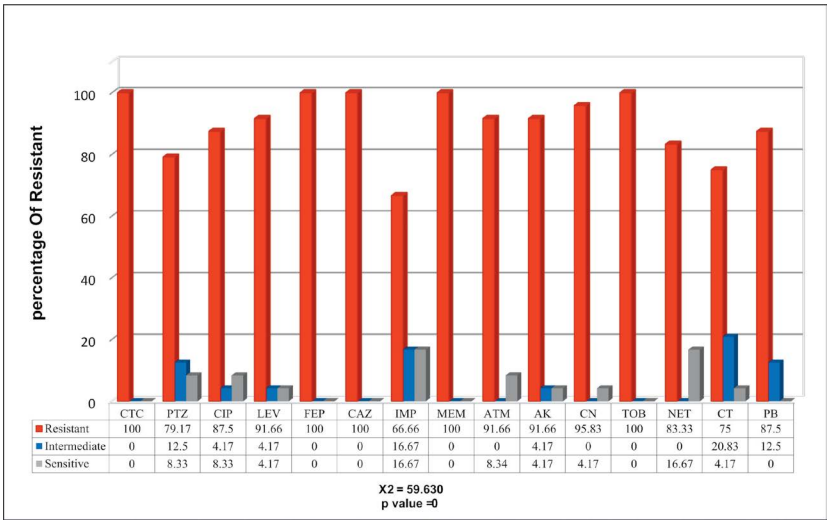

Fig. 1. The distribution of the isolates according to the sampling source

Table 2. The thermocycler reaction conditions

Gene	Time and temperature (°C) Conditions used					No. of cycles
	Initial denaturation	Denaturation	Annealing	Extension	Final extension	
bla_{KPC}	94/5 min	94/30s	57/30s	72/45s	72/10min	30
bla_{GES}	94/5 min	94/30s	59/30s	72/1.5min	72/7min	35
bla_{OXA-48}	94/5 min	94/5min	57/30s	72/30min	72/5min	35
bla_{SME}	94/10 min	94/30s	60/30s	72/50s	72/5min	36

were motile Gram –ve short rods. The results of the biochemical tests showed that the bacteria were positive for catalase, oxides, citrate, motility, and gelatin liquefaction. The API 20 E and Vitek 2 system showed that 100 of the isolates belonged to *P. aeruginosa*, Fig. 1.

With different severity of resistance by percentages of isolates to various antibiotics, only 24% of the isolates displayed resistance against carbapenem, Fig. 2.

The results of the SCM-related MPCR showed the occurrence of the bla_{GES}, bla_{SME}, bla_{KPC}, and bla_{OXA-48} genes in 20 (83.3%), 19 (79.1%), 11 (45.8%), 18 (75%), respectively, of the isolates, Figs. 3, 4, 5, and 6.

The phylogenetic study analysis revealed high, 99%, identity with NCBI-related world strains, table 3 and Fig. 7.

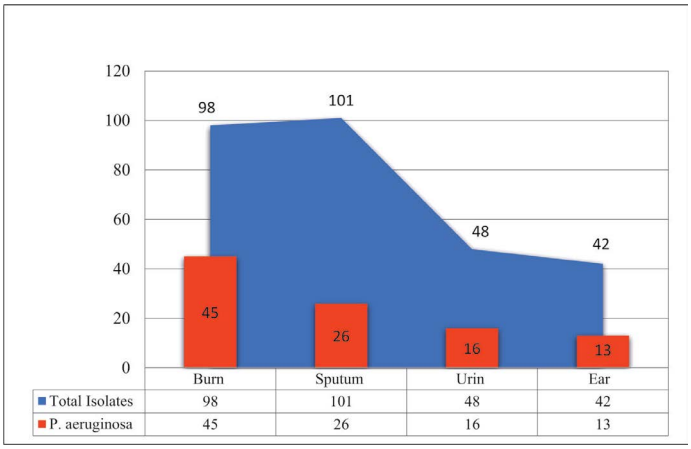

Fig. 2. The percentages of the sensitivity test for the resistant *P. aeruginosa* isolates to carbapenem based on the CLSI (Institute-CLSI, 2017)

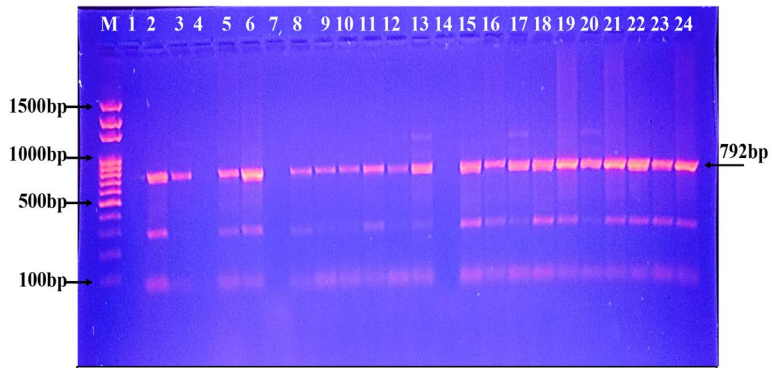

Fig. 3. Agarose gel electrophoresis of the bla_{GES} gene the resistant *P. aeruginosa* isolates to carbapenem. M: ladder, 1500-100bp. Other lanes: positive amplification, 792bp

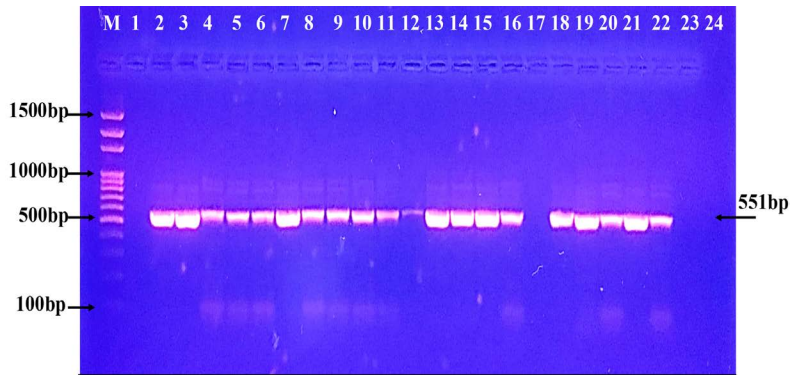

Fig. 4. Agarose gel electrophoresis of the *bla*$_{SME}$ gene the resistant *P. aeruginosa* isolates to carbapenem. M: ladder, 1500-100bp. Other lanes: positive amplification, 551bp

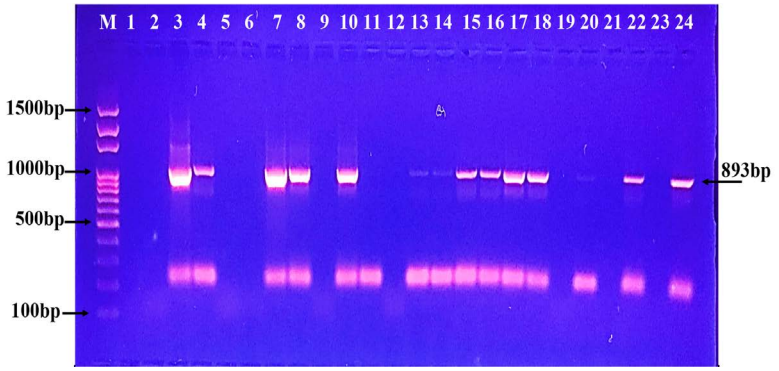

Fig. 5. Agarose gel electrophoresis of the *bla*$_{KPC}$ gene the resistant *P. aeruginosa* isolates to carbapenem. M: ladder, 1500-100bp. Other lanes: positive amplification, 893bp

Table 3. The NCBI-BLAST Homology Sequence identity for the current study isolates of the *P. aeruginosa* resistant to carbapenem

Pseudomonas aeruginosa local isolate	β-lactamase resistance gene	Genbank Accession number	NCBI-BLAST Homology Sequence identity			
			Isolate	Genbank Accession number	(%)	SNP
P. aeruginosa isolate No.6	*bla*$_{GES-39}$	MN182483	*Pseudomonas aeruginosa* strain 153021	MH780083.1	99%	C/A, C/G
P. aeruginosa isolate No.22	*bla*$_{GES-39}$	MN182482	*Pseudomonas aeruginosa* strain 153021	MH780083.1	99%	T/A
P. aeruginosa isolate No.2	*bla*$_{SME-1}$	MN182490	Serratia marcescens S6	NG_050134.1	99%	G/T
P. aeruginosa isolate No.15	*bla*$_{SME-1}$	MN182491	Serratia marcescens S6	NG_050134.1	99%	G/T
P. aeruginosa isolate No.8	*bla*$_{KPC-5}$	MN182497	*Pseudomonas aeruginosa* PR280	NG_049259.1	99%	T/A , G/C, G/T
P. aeruginosa isolate No.1	*bla*$_{OXA-48}$	MN182480	*Pseudomonas aeruginosa* strain DMC-30b	MH168523.1	99%	G/A, C/G
P. aeruginosa isolate No.13	*bla*$_{OXA-48}$	MN182481	*Pseudomonas aeruginosa* strain DMC-30b	MH168523.1	99%	G/A, A/T

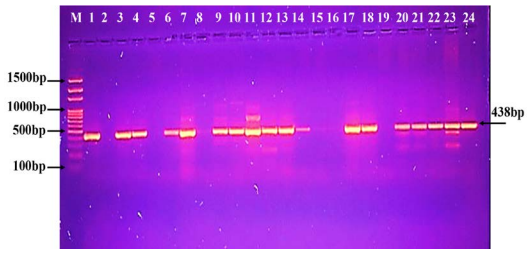

Fig. 6. Agarose gel electrophoresis of the *bla* (OXA-48) gene the resistant *P. aeruginosa* isolates to carbapenem. M: ladder, 1500-100bp. Other lanes: positive amplification, 438bp

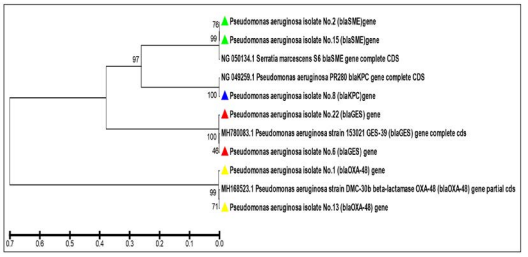

Fig. 7. The phylogenetic tree based on the partial gene sequencing of the serine carbapenemase genes in the resistant *P. aeruginosa* isolates to carbapenem

DISCUSSION

The results showed that the highest percentage of *P. aeruginosa* isolates was from the burn swabs (45.91%). Our results agreed with (de Almeida *et al.*, 2017). who detected that *P. aeruginosa* bacteria were found to be the predominant bacteria in burns. Skin is considered as one of the main mechanical immune barriers that provide protection support against pathogens; however, any destruction facing this barrier may leave the body prone to these opportunistic microorganisms encouraging for nosocomial infections of burns (Russotto *et al.*, 2015; Sousa *et al.*, 2018). The results of the study showed that the most frequent isolated bacteria from burns were the carbapenem-resistant *P. aeruginosa* (66.67%) recovered from admitted burnt patients who were receiving treatment in the Burn Hospital, Al-Diwaniyah City. This result came in agreement with (Al-Shara, 2013). who revealed that carbapenem-resistant *P. aeruginosa* isolates were the dominant bacteria isolated from burns.

KPC enzymes are believed to be as one of the most common Class (A) carbapenemases in the world mainly recognized in *Klebsiella pneumoniae* and *Enterobacteriaceae* (Francis *et al.*, 2012). In the *P. aeruginosa* bacteria, the enzymes were first identified in the United States of America in 2010 showing their gene association with the plasmids, allowing them to move horizontally between bacterial species (Poirel *et al.*, 2011). In conducted studies from Iraq, the coding genes of these enzymes were not identified in *P. aeruginosa* bacteria (Al-Shara, 2013; Mushtak, Shahad and Amer, 2018). Thus and according to the best of our knowledge, the results of the present study reveal for the first time the identification of the *bla* (KPC) gene in *P. aeruginosa* bacteria isolated from Iraq.

The SME-coding gene was first found on a chromosome in *Serratia marcescens* in England (1982) (Queenan and Bush, 2007). The enzyme included three types: SME-1, SME-2, and SME-3, identified in different parts of the United States (Queenan and Bush, 2007; Walther-Rasmussen and Hכiby, 2007). While these enzymes were not previously determined in our country, our results were contrasted with the results of local and global studies to investigate these enzymes in *P. aeruginosa* isolates (Al-Shara, 2013; Khorvash *et al.*, 2017).

The gene bla_{GES} was the most prevalent; however, the gene has different types such as GES-2 and GES 6, GES-14 and GES-20 (Naas, Dortet and I Iorga, 2016; Van Duin and Doi, 2017). The coding genes of these enzymes were detected to be carried on a plasmid, allowing for their transferring between bacterial strains and species (Van Duin and Doi, 2017). GES enzymes were identified in *P. aeruginosa* and *A. bumanni* in South Africa, Greece, Japan, France, and Korea (Patel and Bonomo, 2013).

In the current work, carbabenemase Class D was identified in *P. aeruginosa* isolates via the detection of the bla_{OXA-48} gene. The OXA-48 activity is not limited to carbapenem but is a multi-task enzyme that acts on oxacillin, cloxacillin, and penicillin (Jayol *et al.*, 2016). The enzyme cannot be inhibited by EDTA salt or β-lactams such as Amoxicillin-clavulanic acid (Drawz and Bonomo, 2010) resulting in limited treatment options provided to patients. The OXA-48 enzyme was discovered in bacterial isolates in Egypt, Sudan, Algeria, Tunisia, and France (Nordmann, Naas and

Poirel, 2011; Jayol *et al.*, 2016; Mohamed *et al.*, 2018) To the best of our knowledge, there is no studies explored the presence of such enzymes in *P. aeruginosa* isolates from Iraq. The present study examined the detection of the coding gene of this enzyme *bla* (OXA-48) in the clinical isolates of carbapenem-resistance *P. aeruginosa*.

The phylogenetic study analysis revealed high, 99%, identity with NCBI-related world strains. This gives valued information that our and the world strains might have descended from the same ancestors or evolved to have those resistance genes via horizontal gene transfer.

CONCLUSION

According to the best of our knowledge, this is the first study in Iraq that detects a group of encoding genes of serine carbapenemase in carbapenem-resistant isolates. The current work results may enhance developing better control strategies against *P. aeruginosa* by targeting these genes.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors have made substantial contribution to the work and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not declared.

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