Microbes and Infectious Diseases 2024; Article-In-Press, **DOI:** 10.21608/mid.2024.279851.1883

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Predominance of *bla*$_{KPC}$ carbapenemase gene in multi-drug resistant *Pseudomonas aeruginosa* isolated from ICUs and surgical wards in Egypt

Noha A. Hassuna [1], Nehal E. Koriem [2], Shaimaa H Zaki [1]*, Medhat Abd El Fattah [2], Amani Mohammed Abdelmoaty [3], Reham A. Ibrahem [4]

1- Medical Microbiology and Immunology Department, Faculty of Medicine, Minia University, Minia, Egypt.

2- Botany and Microbiology, Department, Faculty of Science, Beni-Suef University, Beni Suef, Egypt.

3- Pharmaceutics, Faculty of Pharmacy, Beni-Suef University, Beni-Suef University, Beni Suef, Egypt

4- Microbiology and Immunology Department., Faculty of Pharmacy, Minia University, Minia, Egypt.

ARTICLE INFO

Article history:
Received 6 April 2024
Received in revised form 3 May 2024
Accepted 7 May 2024

Keywords:
P. aeruginosa
mCIM
MHT
Carbapenemases
PCR

ABSTRACT

Background: The last-resort classes of antibiotics with the best success in treating severe *Pseudomonas aeruginosa (P. aeruginosa)* infections caused by drug-resistant *P. aeruginosa* are carbapenems. So, we aimed to assess the frequency of carbapenem-resistant genes in *P. aeruginosa.* **Methods**: Samples isolated from critically ill patients at Intensive Care Units (ICUs) and surgical wards in Minia, Egypt were examined for imipenem-resistance phenotypically by Modified Hodge test (MHT) and Modified carbapenem inactivation method (mCIM) test and genotypically by PCR. **Results:** Fifty isolates (64%) of *P. aeruginosa* were resistant to carbapenems (imipenem and meropenem), of which: 16 (32%) were (MHT) positive and 26 (52 %) were (mCIM)-positive. Regarding resistance genes, *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{KPC}$ were detected in 14%, 20%, and 48% of the carbapenem resistant *Pseudomonas aeruginosa* (CRPA) isolates; respectively, while *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{NDM-1}$ and *bla*$_{SPM-1}$ were not detected. **Conclusion:** There is a high prevalence of multi-drug resistant (MDR) strains of *P. aeruginosa.* Doctors should pay attention to carbapenem resistance. mCIM test was much more sensitive than MHT for phenotypic detection of CRPA isolates. *bla*$_{KPC}$ gene was the most frequently detected gene.

Introduction

Gram-negative *Pseudomonas aeruginosa (P. aeruginosa)* is a major health hazard because it can cause healthcare-associated infections among critically ill patients like immunosuppressed patients. It is a common cause of sepsis, ventilator-associated pneumonia, wound infection, and urinary tract infection, particularly in intensive care units (ICUs) [1]. The emergence of multidrug-resistant (MDR) or extensively drug-resistant strains (XDR) of *P. aeruginosa* became a complex challenge and has put the physicians in a complicated situation owing to the reduced number of antibiotic treatment options available leading to failure or delay of antimicrobial treatment, as well as the increase in mortality rate especially with the presence of carbapenem resistant *Pseudomonas aeruginosa* (CRPA) [2].

DOI: 10.21608/MID.2024.279851.1883

* *Corresponding author:* Shaimaa Hassouna Zaki
E-mail address: *shimaa.minia@gmail.com*

© 2020 The author (s). Published by Zagazig University. This is an open access article under the CC BY 4.0 license **https://creativecommons.org/licenses/by/4.0/.**

Carbapenems are broad-spectrum β-lactam antibiotics which are usually considered the last choice for antibiotic therapy to treat serious infections caused by *P. aeruginosa*. Other antimicrobials like colistin and tigecycline may be used in case of carbapenem resistance; however, they have poor efficacy and/or high toxicity [3]. Unfortunately, carbapenems are frequently misused and are becoming ineffective due to the development of many resistance mechanisms produced by gram-negative bacteria [4]. One of the main mechanisms of carbapenem-resistance is the acquisition of metallo beta-lactamases (MBLs) such as bla_{VIM}, bla_{IMP}, bla_{GIM}, bla_{SPM}, and bla_{DIM}, as well as other carbapenemases including bla_{KPC} and bla_{OXA} genes [5].

This resistance has multiple causes, including increased efflux system expression, enzyme production, decreased pore expression, decreased expression of external membrane proteins, and increased topoisomerase enzyme. Mutated genes that produce the carbapenemase enzyme can also cause carbapenem resistance. These genes are divided into four types according to the Ambler classification system based on the sequence of amino acids. Class B (Imipenemases (IMP), Verona integron-encoded metallo-beta-lactamase (VIM) is referred to as a metallo-beta-lactamase because it requires zinc to function, whereas classes A (*Klebsiella pneumoniae* carbapenemase (KPC)) and D oxacillin-hydrolyzing enzyme (OXA) act by a serine-based mechanism. The most prevalent types of carbapenemase in the world were thought to be KPC and VIM [6].

Detection of carbapenemase in *P. aeruginosa* strain is of extreme significance to evade hospital-acquired resistant infections. The availability of accurate and affordable carbapenemase detection techniques may encourage laboratories to investigate this issue and contribute to the prevention of a serious threat of bacterial antibiotic resistance.

Methods

In this cross-sectional study, 310 samples were obtained from patients admitted to ICUs, Neurosurgery, and Surgery Departments in the period between August 2017 and October 2020. Various clinical specimens, i.e. sputum, bronchial lavage, wound fluid, and pus, were collected under complete aseptic conditions. *Pseudomonas aeruginosa* isolates were identified using standard microbiological tests. All experimental protocols were approved by the Ethics Committee of the Faculty of Pharmacy, Minia University. Written informed consent was obtained from the patients prior to data collection. The study was carried out as per the Helsinki declarations.

Bacterial isolation and identification

The bacterial strains were isolated from sputum, wound fluid, pus, and bronchial lavage. Each sample was placed in a sterile container, put in an ice pack box, and transported maximally within 2 hours to the laboratory for processing. The bacterial isolates were identified by traditional biochemical tests including citrate, triple sugar iron (TSI), oxidase, catalase, indole, and Voges-Proskauer (VP) test. Finally, the isolates were stored at -20°C in brain heart infusion (BHI) media containing 20% glycerol [7].

Antimicrobial susceptibility

The antibiotic susceptibility tests were performed on Muller-Hinton agar by using the Kirby-Bauer disc diffusion method according to the CLSI 2018 guidelines [8]. The following antibiotics were used for *P. aeruginosa:* ciprofloxacin (5μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), ceftazidime (30 μg), amikacin (30 μg), gentamicin (10 μg), and piperacillin/tazobactam (110/10 μg). Zone diameters were measured according to CLSI recommendations.

Phenotypic detection of carbapenemases production

Modified Hodge test (MHT) and Modified carbapenem inactivation method (mCIM) test, were performed for all imipenem-resistant isolates.

Modified Hodge test (MHT)

The production of carbapenemases in *P. aeruginosa* was primarily detected by using MHT. A 0.5 McFarland dilution of the *Escherichia coli* ATCC 25922 in 5 ml of broth or saline was prepared. A 1:10 dilution was streaked as a lawn onto a Mueller Hinton agar plate. A 10 μg imipenem disk was placed in the center of the test area. Test organism (CRPA) and quality control organism (MHT Negative *Klebsiella pneumonia* ATCC1706) were streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 37°C for 24 hours. After 24 hours, the MHT Positive test showed a clover leaf-like indentation of the *Escherichia coli* 25922 growing along the test organism growth streak within the disk diffusion zone. MHT Negative test showed no

growth of *Escherichia coli* 25922 along the test organism growth streak within the disk diffusion [9].

Modified carbapenem inactivation method (mCIM)

This method was used to confirm MHT as it is highly sensitive in the detection of carbapenemases than MHT [10]; Suspension of 1*μl* loopful of the test strain was emulsified in 2 ml trypticase soy broth (TSB). The bacterial suspension was vortexed for 10 to 15 seconds. Next, a 10-µg imipenem disk was aseptically added into the bacterial suspension. The tube was then incubated for 4 hours at 37C. Half MF suspension of the indicator strain *E. coli ATCC25922* was prepared by the direct colony suspension method (It was prepared just prior to completion of the 4-hour carbapenem in-activation step). Muller Hinton agar plates were inoculated using the procedure for standard disk diffusion susceptibility testing The imipenem disk was then removed from the TSB bacterial suspension using a 10-µl inoculating loop; the loop was dragged along the edge of the tube during removal to remove excess liquid, and the disk was placed onto the inoculated MHA plate, which was then incubated in an inverted position for 18-24 hours at 37C°. Zone diameter (≤ 15 mm) around the disk indicates positive results and explains the ability of the test organism to produce carbapenemases, (16-19 mm) indicates intermediate results, and(≥ 20mm) indicates negative results [11].

Molecular detection of resistance mechanisms

DNA extraction

The DNA was prepared by using the boiling method by centrifuging 1.5 ml of the bacterial broth for 5 minutes at 11.000 rpm. The supernatant was eliminated, and the pellet was suspended and mixed well with 200 microliters of molecular biology-grade water. Tubes were boiled at 100C° in a water bath for 20 minutes. Then quickly cooled in ice to block the reaction. Samples were centrifuged for 2 minutes at 14.000 rpm. The supernatant was stored at -20 C° and then used for DNA amplification [12].

Amplification of carbapenemase-encoding genes by PCR.

Conventional PCR reactions were performed by using thermal cycler (Biometra, UNO II). Specific primers for *P. aeruginosa* resistance genes bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM-1}, bla_{KPC}, bla_{NDM-1} and bla_{SIM} (**Table 1**).

Amplification of DNA was performed in 25 µl reactions, using My Taq ™ Red Master Mix; (12.5 µl master mix, 2 µl of DNA template, 1 µl of each forward and reverse primers, and 8.5 µl of sterile water). The reaction conditions for each gene are listed in **table (2)**.

Detection of PCR products by agarose gel electrophoresis.

Products of the PCR reaction were separated on 1 %-1.5 % agarose gel stained by 0.5 µg/ml ethidium bromide and bands were visualized under UV light.

Statistical analysis

Descriptive statistics and data are presented as frequency and percentage. For analytical statistics, the Chi-square test was used as a significance test to compare quantitative variables, with a P value ≤ 0.05 indicating high significance. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy analysis were determined to assess the performance of MHT and mCIM in the identification and distinguish carbapenemase using PCR results as a gold standard. Statistical analyses were performed using SPSS 22.0. Cohen' kappa <0.4, poor consistency; Cohen' kappa=0.4–0.75, a fair degree of consistency; Cohen' kappa >0.75, excellent consistency.

Results

A total of 78 (25%) isolates of *P.aeruginosa* were recovered from various clinical samples during the study period (August 2017 to October 2020). Out of 78 isolates of *P. aeruginosa*, (66.7%) were recovered from males and (33.3%) from females, the mean age was 48.8 with *SD* ± 14.4. Most *P. aeruginosa* isolates were recovered from intensive care units (60.3%) and (39.7%) from various Surgical Departments. Most *P. aeruginosa* isolates were recovered from pus and wound fluid (43.6%), sputum (25.6%), urine (16.7%), and (14.1 %) from bronchial lavage. Thirty-two percent (32.1%) of *P. aeruginosa* isolates were recovered from patients on mechanical ventilation, and (14.1%) were from patients with urinary catheters. Moreover (42.3%) recovered from diabetic patients, (21.8%) were from renal failure patients and (10.3%) were from cancer patients **(Table 3).**

Among 78 isolates of *P. aeruginosa*, carbapenem resistance was detected in 50 isolates by disc diffusion method (64%). Among other antipseudomonal antibiotics tested in *P. aeruginosa*

isolates, the maximum resistance was observed for piperacillin/tazobactam (100%), followed by aztreonam and cefepime (97%), ciprofloxacin (93%), amikacin (92%) and ceftazidime (88%) and gentamicin (58%). Regarding the antibiotic and sensitivity results MDR strains were 50 isolates (64%) as all the CRPA strains exhibit resistance to at least three groups of tested antibiotics including carbapenems.

Of 50 CRPA isolates, MHT and mCIM were positive in 16 (32%) and 26 (52%), respectively **(Figure 1)**. Out of 50 CRPA, only 48%, 20%, and 14% were harboring bla_{KPC}, bla_{VIM}, bla_{IMP} genes, respectively. While bla_{SIM}, bla_{GIM}, bla_{SPM-1}, and bla_{NDM} genes were not detected in our study.the photos of gel electrophoresis showing the bands of the resistance genes in **figure (2)**.

In total, there were 39 (50%) CRPA detected by PCR. There were 3 strains with co-existence of the 3 found genes, 2 strains with co-existence of bla_{KPC} and bla_{IMP}, and only 1 sample was positive for 2 genes bla_{VIM} and bla_{IMP} While the other 33 samples were only positive for one gene. The effect of demographic factors on resistance to carbapenem and gene distribution among *P.aeruginosa* isolates **(Tables 4,5)**.

The sensitivity of mCIM test was higher than the sensitivity of MHT, as we found that the sensitivity of mCIM to detect bla_{KPC}, bla_{VIM}, and bla_{IMP} was (79.2%, 60%, 57.1%) respectively and MHT was (45.8%, 50%, 42.9%) for the same genes respectively. The specificity of MHT was (80.8%, 72.5%, and 69.8%) for bla_{KPC}, bla_{VIM}, and bla_{IMP} respectively, and specificity of mCIM was (73.1%, and 50%. 48.8%) for the same genes respectively **(Table 6)**.

By the analysis of the concordance between MHT and mCIM, we found that there is a significant agreement between MHT and mCIM with PCR in the detection of group A β-lactamase (bla_{KPC}) Kappa (0.26 and 0.52) respectively and (*P* 0.044 and <0.001) respectively. According to Kappa test, there is no- concordance between MHT and mCIM with PCR in the detection of class b β-lactamase (bla_{VIM} and bla_{IMP}) **(Table 7)**.

Table 1. Primers used for PCR reaction.

Gene	Primer sequence	Amplicon size (bp)	Ref
bla_{KPC}	F (5' -TGTCACTGTATCGCCGTC-3') R(5'-CTCAGTGCTCTACAGAAAACC-3')	1.011	[13]
bla_{SPM-1}	F(5'-CCTACAATCTAACGGCGACC-3') R(5'-TCGCCGTGTCCAGGTATAAC-3')	649	[14]
bla_{NDM-1}	F(5'-GGTTTGGCGATCTGGTTTTC-3') R(5'-CGGAATGGCTCATCACGATC-3')	621	[15]
bla_{IMP}	F(5'-GGAATAGAGTGGCTTAACTCTC-3') R(5'-CGAATGCGCACCAG-3')	232	[16]
bla_{VIM}	F (5'-TGG TGT TTG GTC GCA AT-3') R (5'-CGA ATG CGC ACC AG -3')	390	[16]
bla_{GIM}	F(5'-TCGACACACCTTGGTCTGAA-3') R(5'-AACTTCCAACTTTGCCATGC-3')	477	[17]
bla_{SIM}	F(5'-TACAAGGGATTCGGCATCG-3') R(5'-TAATGGCCTGTTCCCATGTG-3')	570	[17]

Table 2. Conditions for PCR reactions:

Gene	**Cycles**	**Initial denaturation**		**Denaturation**		**Annealing**			**Extension**
		Time	Temp	Time	Temp	Time	Temp	Time	**Temp**
bla KPC	30	5 min	95 C°	1 min	94 C°	1min	55 C°	1 min	**72 C°**
bla SPM-1	30	5 min	95 C°	1min	95 C°	1 min	40 C°	1 min	**68 C°**
bla NDM-1	36	10 min	94 C°	30 sec	94 C°	40 sec	52 C°	50sec 5 min	**72 C°** **72 C°**
bla IMP *bla* VIM	30	2 min	95 C°	30	95 C°	30	48 C°	30 sec 2 min	**72 C°** **72 C°**
bla GIM *bla* SIM	36	5 min	94 C°	30 sec	94 C	40 sec 50 sec	52 C° 72 C°	5 min	**72 C°**

Table 3. Demographic characteristics of *P.aeruginosa* isolates.

		Percentage of *P. aeruginosa* N=78
Age	*Range* *Mean ± SD*	(15-75) 48.8±14.4
Gender	*Male* *Female*	52(66.7%) 26(33.3%)
Source of samples	*Pus and Wound fluid* *Sputum* *Bronchial lavage* *Urine*	34(43.6%) 20(25.6%) 11(14.1%) 13(16.7%)
Underlying disease	*No* *DM* *Renal* *Cancer*	20(25.6%) 33(42.3%) 17(21.8%) 8(10.3%)
Admission site	*ICU* *Surgical department*	47(60.3%) 31(39.7%)
Related devices	*None* *Ventilator* *Urinary catheter*	42(53.8%) 25(32.1%) 11(14.1%)

SD: standard deviation, DM: diabetes mellitus, ICU: intensive care unit.

Table 4. Relation between some demographic factors and carbapenem resistance among *P. aeruginosa* isolates.

Related devices					
		None (I)	**Ventilator (II)**	**Urinary catheter (III)**	***p* value**
		N=42	**N=25**	**N=11**	
Carbapenem	*Sensitive* *Resistant*	10(23.8%) 32(76.2%)	10(40%) 15(60%)	8(72.7%) 3(27.3%)	***0.009****

Underlying diseases						
		No (I)	**DM (II)**	**Renal (III)**	**Cancer (IV)**	***p* value**
		N=20	**N=33**	**N=17**	**N=8**	
Carbapenem	*Sensitive* *Resistant*	14(70%) 6(30%)	9(27.3%) 24(72.7%)	4(23.5%) 13(76.5%)	1(12.5%) 7(87.5%)	***0.003****

Source of samples						
		Pus and Wound fluid (I)	**Sputum (II)**	**Bronchial lavage (III)**	**Urine (IV)**	***p* value**
		N=34	**N=20**	**N=11**	**N=13**	
Carbapenem	*Sensitive* *Resistant*	6(17.6%) 28(82.4%)	4(20%) 16(80%)	7(63.6%) 4(36.4%)	11(84.6%) 2(15.4%)	***<0.001****

Admission site				
		ICU	**Surgical department**	***p* value**
		N=47	**N=31**	
Carbapenem	*Sensitive* *Resistant*	12(25.5%) 35(74.5%)	16(51.6%) 15(48.4%)	***0.019****

Chi square test ,: Significant level at p value < 0.05*

Table 5. Relation between some demographic factors with phenotype and gene distribution among CRPA isolates.

		Related devices				***p* value**
		None (I)	**Ventilator (II)**	**Urinary catheter (III)**		
		N=31	**N=15**	**N=3**		
MHT	*-Ve*	23(71.9%)	9(60%)	2(66.7%)		*0.717*
	+Ve	9(28.1%)	6(40%)	1(33.3%)		
Mcim	*-Ve*	18(56.3%)	5(33.3%)	1(33.3%)		*0.298*
	+Ve	14(43.8%)	10(66.7%)	2(66.7%)		
bla$_{KPC}$	*-Ve*	19(59.4%)	5(33.3%)	2(66.7%)		*0.218*
	+Ve	13(40.6%)	10(66.7%)	1(33.3%)		
bla$_{VIM}$	*-Ve*	29(90.6%)	10(66.7%)	1(33.3%)		***0.018******
	+Ve	3(9.4%)	5(33.3%)	2(66.7%)		
bla$_{IMP}$	*-Ve*	26(81.3%)	14(93.3%)	3(100%)		*0.415*
	+Ve	6(18.8%)	1(6.7%)	0(0%)		
		Underlying diseases				***p* value**
		No (I)	**DM (II)**	**Renal (III)**	**Cancer (IV)**	
		N=6	**N=24**	**N=13**	**N=7**	
MHT	*-Ve*	5(83.3%)	15(62.5%)	9(69.2%)	5(71.4%)	*0.794*
	+Ve	1(16.7%)	9(37.5%)	4(30.8%)	2(28.6%)	
Mcim	*-Ve*	5(83.3%)	8(33.3%)	7(53.8%)	4(57.1%)	*0.140*
	+Ve	1(16.7%)	16(66.7%)	6(46.2%)	3(42.9%)	
bla$_{KPC}$	*-Ve*	2(33.3%)	11(45.8%)	9(69.2%)	4(57.1%)	*0.420*
	+Ve	4(66.7%)	13(54.2%)	4(30.8%)	3(42.9%)	
bla$_{VIM}$	*-Ve*	6(100%)	20(83.3%)	9(69.2%)	5(71.4%)	*0.402*
	+Ve	0(0%)	4(16.7%)	4(30.8%)	2(28.6%)	
bla$_{IMP}$	*-Ve*	6(100%)	18(75%)	12(92.3%)	7(100%)	*0.175*
	+Ve	0(0%)	6(25%)	1(7.7%)	0(0%)	
		Source of samples				***p* value**
		Pus and Wound fluid (I)	**Sputum (II)**	**Bronchial lavage (III)**	**Urine (IV)**	
		N=28	**N=16**	**N=4**	**N=2**	
MHT	*-Ve*	18(64.3%)	13(81.3%)	2(50%)	1(50%)	*0.501*
	+Ve	10(35.7%)	3(18.8%)	2(50%)	1(50%)	
Mcim	*-Ve*	13(46.4%)	9(56.3%)	2(50%)	0(0%)	*0.509*
	+Ve	15(53.6%)	7(43.8%)	2(50%)	2(100%)	
bla$_{KPC}$	*-Ve*	14(50%)	9(56.3%)	1(25%)	2(100%)	*0.365*
	+Ve	14(50%)	7(43.8%)	3(75%)	0(0%)	
bla$_{VIM}$	*-Ve*	24(85.7%)	13(81.3%)	3(75%)	0(0%)	***0.034******
	+Ve	4(14.3%)	3(18.8%)	1(25%)	2(100%)	
bla$_{IMP}$	*-Ve*	23(82.1%)	14(87.5%)	4(100%)	2(100%)	*0.717*
	+Ve	5(17.9%)	2(12.5%)	0(0%)	0(0%)	
		Admission site				**P value**
		ICU	**Surgical Department**			
		N=35	**N=15**			
MHT	*-Ve*	26(74.3%)	8(53.3%)			*0.146*
	+Ve	9(25.7%)	7(46.7%)			
Mcim	*-Ve*	17(48.6%)	7(46.7%)			*0.902*
	+Ve	18(51.4%)	8(53.3%)			
bla$_{KPC}$	*-Ve*	17(48.6%)	9(60%)			*0.459*
	+Ve	18(51.4%)	6(40%)			
bla$_{VIM}$	*-Ve*	25(71.4%)	15(100%)			***0.021******
	+Ve	10(28.6%)	0(0%)			
bla$_{IMP}$	*-Ve*	32(91.4%)	11(73.3%)			*0.091*
	+Ve	3(8.6%)	4(26.7%)			

*Chi square test, *: Significant level at P value < 0.05*

Table 6. Results of sensitivity, specificity, NPV, and PPV in MHT versus mCIM test in CRPA.

PCR	**MHT**					**mCIM**				
	SE (%)	SP (%)	PPV (%)	NPV (%)	accuracy	SE (%)	SP (%)	PPV (%)	NPV (%)	accuracy
***bla*KPC**	45.8	80.8	71.1	61.8	64	79.2	73.1	73.1	79.2	76
***bla*VIM**	50	72.5	31.3	85.3	68	60	50	23.1	83.3	52
blaIMP	42.9	69.8	18.8	88.2	66	57.1	48.8	15.4	87.5	50

∗NPV: negative predictive values. ∗PPV: positive predictive value. *SE: sensitivity.*SP: specificity.

Table 7. Concordance between phenotypic and genotypic methods.

		MHT		***Kappa***	***p value***
		-Ve	**+Ve**		
***bla* KPC**	*-Ve* *+Ve*	21(61.8%) 13(38.2%)	5(31.3%) 11(68.8%)	***0.269***	***0.044****
***bla* VIM**	*-Ve* *+Ve*	29(85.3%) 5(14.7%)	11(68.8%) 5(31.3%)	*0.184*	*0.172*
***bla* IMP**	*-Ve* *+Ve*	30(88.2%) 4(11.8%)	13(81.3%) 3(18.8%)	*0.082*	*0.507*
		mCIM		***Kappa***	***p value***
		-Ve	**+Ve**		
***bla* KPC**	*-Ve* *+Ve*	19(79.2%) 5(20.8%)	7(26.9%) 19(73.1%)	***0.521***	***<0.001****
***bla* VIM**	*-Ve* *+Ve*	20(83.3%) 4(16.7%)	20(76.9%) 6(23.1%)	*0.063*	*0.571*
***bla* IMP**	*-Ve* *+Ve*	21(87.5%) 3(12.5%)	22(84.6%) 4(15.4%)	*0.028*	*0.769*

*Kappa test, *: Significant level at P value < 0.05*

Figure 1. Phenotypic detection for carbapenemases by MHT and mCIM in CRPA. A: MHT in CRPA: 1: negative control, 2- negative MHT, 3- Positive MHT. B: Negative mCIM against meropenem and imipenem in CRPA. C: Positive mCIM against meropenem and imipenem in CRPA.

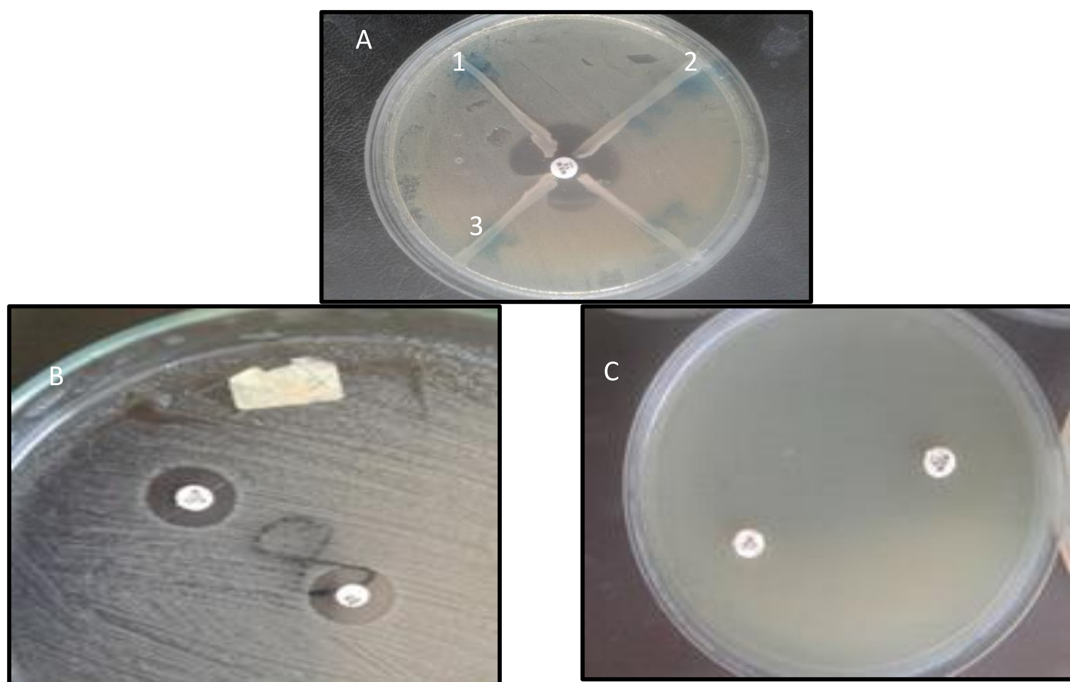
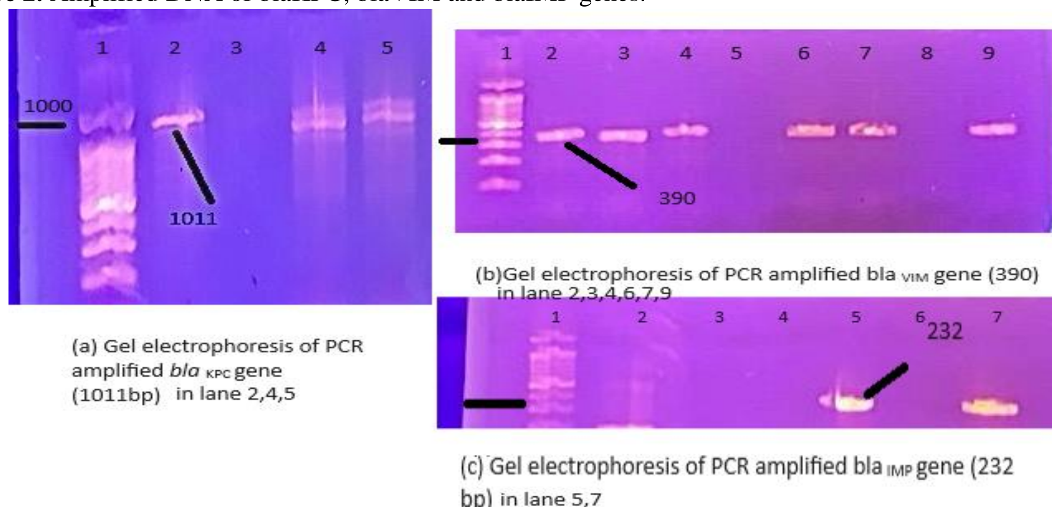


Figure 2. Amplified DNA of blaKPC, blaVIM and blaIMP genes.



Discussion

Carbapenem resistance is a global public health issue that is predominantly found in *P. aeruginosa*. Antibiotic resistance is rapidly spreading, particularly when it is spread through transferable carbapenemase-encoding genes like Metallo β lactamases, resulting in large outbreaks and limiting treatment options [18]. By the analysis of the results of this study, we found that the prevalence of *P. aeruginosa* was (25%) of the total collected samples and this result agrees with **Farhan et al.** [19] which recorded (28.3 %) positive *P. aeruginosa* isolated from Minia hospitals, Egypt.

Resistance rates are increasing among several Gram-negative bacteria, especially *P. aeruginosa*, leading to the emergence of MDR strains and leaving just a small number of antibiotics to choose from. As (64%) of *P. aeruginosa* were MDR in this study as well as in **Farhan et al** who found MDR in 66% [19]. The high resistance of *P. aeruginosa* isolates was observed for piperacillin/tazobactam, aztreonam, cefepime, ciprofloxacin ceftazidime, imipenem, meropenem, and gentamycin. We found that the resistance to those antibiotics was increased by comparison to previous studies concerning the misuse of different antibiotics. As (94.7%) of *P. aeruginosa* were resistant to piperacillin/ tazobactam [20].

Resistance to cefepime was (53.5%) [21]. We noticed that the resistance to ciprofloxacin was increased as it was lower in previous studies by (72.6%) [18]. Resistance to amikacin was (85.7%) by **Walters et al.** [18]. Resistance to ceftazidime was (74.4 %) [13]. Resistance to gentamicin was (43.9%) by **Mahmoud et al**. [22]. Resistance to aztreonam was (83.3%) by **Abd El-Baky et al.** [16] and (37.5%) [21], this difference in resistance patterns among *P. aeruginosa* strains isolated from different regions may be due to the misuse of antibiotics, especially there is an increase in the use of carbapenems to treat infections all over the world, the horizontal gene transfer, and the influence of the environmental conditions [23]. This, along with a lack of information about detection, raises the risk of multidrug-resistant pathogens spreading in hospitals. *P. aeruginosa* was highly resistant to carbapenemases as we found that (64.0%) of *P.aeruginosa* were resistant to imipenem and meropenem. These results resembled those reported in the earlier studies done in Egypt by **Abaza et al.** [24] which recorded (73.7 %) resistant *P.aeruginosa* to both meropenem and imipenem.

P. aeruginosa resistance to carbapenems is significantly impacted by some demographic characteristics; We found that the majority of CRPA isolates were more frequently found in patients admitted to ICUs than in surgical departments, and this difference was statistically significant (p=0.01). Additionally, we discovered that patients who used intrusive devices including mechanical ventilation and urinary catheters were more susceptible to CRPA infection than patients who did not use any of these devices, and this difference was statistically significant (p=0.009). The underlying diseases like hyperglycemia, malignancy, and renal failure might reduce the immune system's defense, rendering diabetics, malignant and renal failure patients more

vulnerable to CRPA infection and this difference was significant (p=0.003), and this was supported by findings from other studies [25]. CRPA were predominantly isolated from pus and wound fluids, followed by sputum sample, the same has been reported by **Vijaya Chaudhari et al.** [26] who reported that wound infection and respiratory tract infections were found to be commonly affected by *P.aeruginosa.*

The observed discrepancies may be explained by variations in resistance screening techniques, antibiotic prescription frequency, and policy. Additionally, the variations may result from genetic alterations in isolates created by unwarranted antibiotic prescriptions in various countries around the world, as well as from an increase of resistance mechanisms, particularly beta-lactamase production, resulting in a wide range of resistance in our country's hospitals.

According to phenotypic tests for the determination of carbapenemase production by using MHT, our result complies with another study done by **Jayalakshmi et al.** [27] recorded (33%) positive MHT. Another study was done by **Falahat et al.** [28] which reported (35%) positive MHT. A higher percentage was found by using the modified carbapenem inactivation method (mCIM) (52%) and this agrees with **Ferjani et al.** [29] who recorded (46.15%) positive mCIM.

The bla_{KPC} gene was the most frequent gene found among CRPA enrolled in this study as it was detected in 24 (48%). The prevalence of the bla_{KPC} gene was highly variant among the previous studies done in different countries as it represented (50%) [30]. In another study done in Arkan, it was (12%). The current elevated incidence may be attributed to the horizontal gene transfer from other bacteria as KPC is carried on mobile elements such as plasmids which are highly transferable elements and this explains their rapid dissemination in hospital environments [31].

The bla_{VIM} gene was detected in 10 (20%) of carbapenem-resistant isolates and the bla_{IMP} gene was detected in 7 (14 %) of resistant isolates. These findings agreed with **El Essawy et al.** [30] who recorded that bla_{VIM} and bla_{IMP} genes were detected in (22.7%), and (18.2%) respectively. However lower incidence of bla_{IMP} according to a study done in Egypt [31]. The co-existence of two or three carbapenemase genes especially MBLs, was observed in many isolates isolated from patients with underlying chronic diseases and mostly admitted to ICU, and those isolates had a very broad spectrum of resistance to antibiotics. The co-existence of bla_{IMP} with bla_{VIM} and/ or bla_{KPC} may indicate that there is no confirmed sole role of the bla_{IMP} gene on carbapenem resistance in the tested strains. To clarify this point, plasmid profiles and plasmid curing experiments may be needed [30]. The bla_{GIM} and bla_{SPM-1} genes were not detected in this study as previously reported by **Abbas et al.** [32]. Our findings showed that mCIM sensitivity is much higher than MHT sensitivity in the identification of carbapenemases, particularly bla_{KPC} (Se79.2%, Sp 73.1%), and both phenotypic tests had a poor agreement with PCR results, except for bla_{KPC} gene determination. This poor concordance may be explained by the two tests' low metallo beta-lactamase specificity and sensitivity, as well as the existence of false-positive results in both tests, as previously indicated by **Carvalhaes et al.** [33] who reported that the false-positive results might be due to the porin loss in the cell wall of bacteria, so these tests are not a suitable method for the identification of MBL producing isolates and PCR is still the most accurate method in detection of carbapenemases.

Conclusions

Our data revealed that *P. aeruginosa* had a great ability of resistance to many antibiotics as it was detected as MDR strains. Carbapenem resistance, especially imipenem, and meropenem was detected in the strains isolated, this was due to the presence of carbapenemases especially the MBLs, which cause serious problems in forcing these pathogens. Most CRPA strains were isolated from ICUs and we believed that they are the potential source of infection in our hospitals. The most accurate method for carbapenemase detection is PCR because of the low sensitivity and specificity of MHT and mCIM. Carbapenemases were extremely complex determinants of resistance as they spread rapidly across the bacterial community creating a threat to the treatment with carbapenems. We noticed that the main mechanisms of resistance in *P. aeruginosa* were the carbapenem hydrolyzing gene encoded by the acquired gene bla_{KPC} followed by bla_{VIM} and bla_{IMP}. Determinant factors like the site of admission, use of mechanical devices, and the presence of underlying diseases had a great influence on the resistance of *P. aeruginosa* to carbapenems.

Declarations

All methods were carried out in accordance with Helsinki declarations. All experimental protocols were approved by the Ethics Committee of the Faculty of Pharmacy,Minia University. Informed consent was obtained from all subjects

Disclosure of potential conflicts of interest: The authors declare that they have no Conflict of interests.

Funding:

None.

Data availability:

All data generated or analyzed during this study are included in this article.

References

1- **Hassuna NA, Darwish MK, Sayed M, Ibrahem RA.** Molecular epidemiology and mechanisms of high-level resistance to meropenem and imipenem in pseudomonas aeruginosa. Infet Drug Resist. 2020; 13: 285-293.

2- **Al-kasaby NM, Zaki M.** Molecular study of Acinetobacter baumannii isolates for metallo-β-lactamases and extended-spectrum-β-lactamases genes in intensive care unit, Mansoura University Hospital, Egypt. Inter J Microbiol. 2017; 2017: 1-6.

3- **Azimi L, Fallah F, Karimi A, Shirdoust M, Azimi T, Sedighi I. Rahbar M, et al.** Survey of various carbapenem-resistant mechanisms of *Acinetobacter baumannii and Pseudomonas aeruginosa* isolated from clinical samples in Iran. I J Bas Medical Sci. 2020; 23: 1396-1400.

4- **Ramadan RA, Gebriel MG, Kadry, HM, Mosallem A.** Carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: characterization of carbapenemase genes and E-test evaluation of colistin-based combinations. Infect Drug Resist. 2018; 11: 1261– 1269.

5- **Kindu M, Derseh L, Gelaw B, Moges F.** Carbapenemase-Producing Non-Glucose-Fermenting Gram-Negative Bacilli in Africa, Pseudomonas aeruginosa and Acinetobacter baumannii, A Systematic Review and Meta-Analysis. Int J Microbiol.2020; 2020: 9461901.

6- **Bakhat S, Taj Y, Hanif F, Faheem MF.** Evaluation of Phenotypic Methods for Detection Of Carbapenem Resistance In Isolates Of Pseudomonas Aeruginosa In A Tertiary Care Hospital. Journal of Bahria university medical and dental college. 2019; 9(3):165-169.

7- **Howard DH.** The preservation of bacteria by freezing in glycerol broth. J Bacteriol. 1956;71(5):625.

8- **CLSI.** M100 Performance Standards for Antimicrobial Susceptibility Testing A CLSI supplement for global application. 30th edition.

9- **Pasteran F, Gonzalez LJ, Albornoz E, Bahr G, Vila AJ, Corso A**. Triton Hodge test: improved protocol for modified Hodge test for enhanced detection of NDM and other carbapenemase producers. J Clin Microbiol. 2016; 54(3): 640–649.

10- **Jing X, Zhou H, Min X, Zhang X, Yang Q, Du S , et al**. The Simplified Carbapenem Inactivation Method (sCIM) for simple and accurate detection of carbapenemase-producing gram-negative bacilli. Front Microbiol. 2018; 30: 9-2391.

11- **Pierce VM, Simner PJ, Lonsway DR, Roe-Carpenter DE, Johnson JK, Brasso WB, et al.** Modified Carbapenem Inactivation Method for Phenotypic Detection of Carbapenemase Production among Enterobacteriaceae. J Clin Microbiol. 2017 Aug;55(8):2321-2333.

12- **Queipo-Ortuño MI, Colmenero JD, Macias M, Bravo MJ, Morata P.** Preparation of

Bacterial DNA Template by Boiling and Effect of Immunoglobulin G as an Inhibitor in Real-Time PCR for Serum Samples from Patients with Brucellosis. Clin & DiagLab Imm. 2008;15(2): 293-296.

13- **Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, et al.** Novel carbapenem- hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumonia.* Antimicrob Agents Chemother. 2001; 45(4):1151-1161.

14- **Gales AC, Menezes LC, Silbert S, Sader HS.** Dissemination in distinct Brazilian regions of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing SPM metallo-β-lactamase. J Antimicrob Chemother.2003; 52: 699–702.

15- **Nordmann P, Naas T, Poirel L.** Global spread of Carbapenemase-producing Enterobacteriaceae.EInfectDis.2011; 17(10):1791-1798.

16- **Adam MA, Elhag WI.** Prevalence of metallo-β-lactamase acquired genes among carbapenems susceptible and resistant Gram-negative clinical isolates using multiplex PCR, Khartoum hospitals, Khartoum Sudan. BMC Infect Dis. 2018; 18(1):668.

17- **Ellington MJ, Kistler J, Livermore DM, Woodford N.** Multiplex PCR for rapid detection of gene s encoding acquired metallo-beta-lactamases. J Antimicrob Chemother. 2007; 59(2):321-2.

18- **Walters M, Grass JE, Bulens SN, Hancock EB, Phipps EC, Muleta D, et al.** Carbapenem-resistant *Pseudomonas aeruginosa* at US emerging infections Program Sites,2015. Emerg Infect Dis. 2019; 25(7): 1281-1288.

19- **Martis M, Leroy S, Blanc V.** Colistin in multi-drug resistant *Pseudomonas aeruginosa* blood-stream infections a narrative review for the clinician.J Infect. 2014; 69: 1-12.

20- **Mahmoud AB, Zahran WA, Hindawi GR, Labib AZ, Galal R.** Prevalence of Multidrug-Resistant *Pseudomonas aeruginosa* in Patients with nosocomial infections at a University Hospital in Egypt, with Special Reference to Typing Methods. J Virol Microbiol. 2013; 1-13.

21- **Farhan SM, Ibrahim RA, Mahran KM, Hetta HF, Abd El-Baky RM.** Antimicrobial resistance pattern and molecular genetic distribution of metallo-β-lactamases producing Pseudomonas aeruginosa isolated from hospitals in Minia, Egypt. Infect Drug Resist. 2019; 12:2125-2133.

22- **Abd El-Baky RM, Masoud SM, Mohamed DS, Waly NG, Shafik EA, Mohareb DA, et al.** Prevalence and Some Possible Mechanisms of Colistin Resistance Among Multidrug-Resistant and Extensively Drug-Resistant Pseudomonas aeruginosa. Infect Drug Resist. 2020 Feb 3; 13: 323- 332.

23- **Dahdouh E, Shoucair SH, Salem SE, Daoud Z.** Mutant prevention concentrations of Imipenem and Meropenem against Pseudomonas aeruginosa and Acinetobacter baumannii. Sci W J. 2014; l: 1-7.

24- **Abaza AF, El-shazly SA, Selim HS, Aly GS.** Metallo-Beta-Lactamase Producing Pseudomonas aeruginosa in a Healthcare Setting in Alexandria, Egypt, Pol J Microbiol.2017; 66(3): 297–308.

25- **Petrova AP, Stanimirova ID, Ivanov IN, Petrov MM, Miteva-Katrandzhieva TM, Grivnev VI, et al.** Carbapenemase Production of Clinical Isolates *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from a

Bulgarian University Hospital. Folia Med (Plovdiv). 2017 Dec 20; 59(4):413-422.

26- **Vijaya C, Sandeep S, Mukesh M.** Antibiotic resistance patterns of *Pseudomonas aeruginosa* in a tertiary care hospital, in Central India. International J.of Med sci and Pub Health.2013; 2(2):386–89

27- **Jayalakshmi S, Pandurangan S.** Performance of Modified Hodge Test for Detection of Carbapenemase Producing Clinical Isolates of Pseudomonas aeruginosa. Int.J.Curr.Microbiol.App.Sci.2016;5(4):127-132.

28- **Falahat S, Shojapour M, Sadeghi A.** Detection of KPC Carbapenemase in *Pseudomonas aeruginosa* isolated from clinical samples using Modified Hodge Test and Boronic Acid Phenotypic methods and Their Comparison with the Polymerase Chain Reaction. Jundishapur J Microbiol. 2016; 9 (9):e27249.

29- **Ferjani. A, Kanzari L,.Boubaker.** I.B.B. Evaluation of three carbapenemase-phenotypic detection methods and emergence of diverse VIM and GES variants among Pseudomonas aeruginosa isolates in Tunisia. Antib 2022; 11(858.

30- **El-Essawy AK, Abu-Shady HM, El Biaa BA.** Prevalence and impact of carbapenemase-encoding genes in clinical isolates of carbapenem-resistant Pseudomonas aeruginosa. Egypt. J. Exp. Biol. (Bot.). 2018; 14(1): 161– 169 (2018)

31- **El-Domany RA, Mohamed E, El-Magd MA. Moustafa WH, Abdeltwab NM.** Emergence of Imipenem-Resistant *Pseudomonas aeruginosa* Clinical Isolates from Egypt Coharboring VIMand IMP Carbapenemases. Microbial Drug Resistance. 2017; 23(6): 682-686(2017).

32- **Abbas HA, El-Ganiny AM, Kamel HA.** Phenotypic and genotypic detection of antibiotic resistance of Pseudomonas aeruginosa isolated from urinary tract infections. Afri Health Sci. 2018; 18(1): 11-21.

33- **Carvalhaes CG, Picao RC, Nicoletti AG Xavier DE, Gales AC**. "Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results." Journal of antimicrobial chemotherapy 2010; 65(2): 249-251.

Hassuna NA, Koriem NE, Zaki SH, Abd El Fattah M, Abdelmoaty AM, Ibrahem RA. Predominance of *bla*$_{KPC}$ carbapenemase gene in multi-drug resistant *Pseudomonas aeruginosa* isolated from ICUs and surgical wards in Egypt. Microbes Infect Dis 2024; Article-In-Press, **DOI:** 10.21608/mid.2024.279851.1883.