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RESEARCH ARTICLE OPEN ACCESS

Emergence of Multidrug-resistant Carbapenemases and MCR-1 Producing *Pseudomonas aeruginosa* in Egypt

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Abstract

***Pseudomonas aeruginosa* is an expedient Gram-negative bacterium, which is characterized by its ability to acquire antimicrobial resistance. In this study, 56 unrepeatable carbapenem-resistant *P. aeruginosa* isolates were gathered from various clinical sources from hospitals in Cairo and Mansoura universities. The isolates exhibited diminished susceptibility towards carbapenems, quinolones, aminoglycosides and chloramphenicol by using disc diffusion method. Carbapenemase production was confirmed among the isolates, where all the 56 *P. aeruginosa* isolates harboured carbapenemase genes including bla_{VIM} (43 isolates), bla_{KPC} (38 isolates), bla_{NDM-1} (17 isolates), bla_{IMP} (16 isolates) and bla_{OXA-48} (15 isolates). Among the isolates, 13 carried only one carbapenemase gene, while 43 isolates carried multiple carbapenemase genes. MCR-1 production was confirmed in 10 of the tested isolates by detecting the *mcr-1* gene encoding for the colistin resistance. Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) evaluation showed that the tested isolates were unrelated to each other. Therefore, this study rises the danger of emergence of MDR *P. aeruginosa* resistant to carbapenems coupled with other antimicrobials including colistin, which is regarded as the last reservoir for the management of infections caused by MDR Gram-negative pathogens. Early inspection of resistance patterns in MDR organisms is an important tool to control and prevent infections via limiting the spread of these pathogens.**

Keywords: Carbapenemase, Colistin, Egypt, Multidrug-resistance, *Pseudomonas aeruginosa*

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INTRODUCTION

Pseudomonas aeruginosa is a threatening human pathogen that is usually linked to nosocomial infections.[1,2] Commonly, this pathogen is multidrug-resistant (MDR) due to its extraordinary capacity to acquire resistance to a wide range of antimicrobials.[3] Usually, carbapenems are considered the first treatment choice against infections brought about by *P. aeruginosa*. Because of the widespread use of carbapenems, there has been a global increase in reports regarding *P. aeruginosa* carbapenem resistant isolates.[4] It is also notable that isolates of *P. aeruginosa* have started to depict resistance to the antibiotics that are used as a last choice for therapy including colistin.[5]

Indeed, the primary mechanism for pathogens to resist carbapenems is the production of carbapenemases, which are mainly plasmid mediated and considered as a type of β-lactamases.[6] Carbapenemases have a wide range of hydrolytic capabilities against antimicrobials including carbapenems, cephalosporins and penicillins.[7,8] Three major types of carbapenemases have been identified to be the source of nosocomial epidemics in *P. aeruginosa* involving the KPC type (class A serine enzymes), the IMP, VIM and NDM-1 types (class B metallo-β-lactamases (MBLs) or metal enzymes) and the OXA-48 type (class D enzymes).[7-11] Class B enzymes are MBLs with zinc in the active site, while class A and D enzymes have a serine-based hydrolytic mechanism.[8-10,12]

The most significant developing mechanisms of β-lactam resistance in *P. aeruginosa* are those arbitrated by extended-spectrum β-lactamases (ESBLs) and MBLs. β-lactamases inhibitors do not inhibit MBLs, which have a significant capacity to hydrolyze carbapenems efficiently and other β-lactams.[10,11,13,14] The KPC type is primarily present on pKpQIL plasmid, which occurs in *Klebsiella pneumonia*.[15] The OXA-48 type is frequently found on carbonen 62-kb IncL/M conjugative plasmids of *Acinetobacter baumannii*.[12]

Notably, polymyxins are typically considered the final treatment line against MDR Gram-negative bacteria. The polycationic peptide called colistin (also named to as polymyxin E) binds to anionic lipopolysaccharide molecules in the outer membrane of Gram-negative cell walls and disrupts them by competing with $Ca2^+$ and $Mg2^+$ cations for these molecules.[16,17] Unfortunately, colistin resistance has recently developed because of the drug misuse.[16] The horizontal transfer of phosphoethanolamine transferase enzyme-encoding gene *mcr-1* was discovered in 2015 and it has a key role in the colistin resistance.[18] This enzyme has the ability to modify the lipopolysaccharides of the outer membrane lipid A of Gram-negative pathogens.[17,19]

The horizontal transfer of plasmid-mediated resistance has two risks. First, the plasmids can confer the resistance to a variety of drugs. Second, plasmids have a stronger capacity to propagate resistance among bacteria than natural mutation.[4-6] The rise of carbapenem-resistant pathogens all over the world with identical mobile genetic elements suggests that genes for carbapenemases have been spread horizontally.[6] The MBL-producing genes bla_{VIM}, bla_{NDM-1} and bla_{IMP} as well as bla_{KPC} (class A) and bla_{OXA-48} (class D) genes can transfer horizontally via plasmids and can spread quickly to other bacteria.[4,5,7,8,10,11,20] The same case is for *mcr*-1, which is plasmid mediated and has the ability to spread quickly over the globe.[17,19]

Based on this context, the objective of the present investigation was to estimate the dominance of MBLs (VIM, NDM-1 and IMP) as well as class serine enzymes (the KPC type and OXA-48 type), which are responsible for carbapenemases activity in the isolates of *P. aeruginosa*. Moreover, to investigate the prevalence of MCR-1 among the isolates. After all, the clonal clustering of the tested isolates was established utilizing (ERIC)-PCR.

MATERIALS AND METHODS

Bacterial isolates and media

This study was carried out after the approval of the research ethics committee of Faculty of Pharmacy, Tanta University, Egypt (code: TP / RE /12-21-M-002). The current study included 56 unrepeatable carbapenem-resistant *P. aeruginosa* isolates. These isolates were gathered from several clinical sources from hospitals in Cairo and Mansoura universities. The examined isolates were collected, identified and stored using the

Table 1. Oligonucleotide primers used in this study

Gene name	Primer Type	Sequence	Amplicon size (bp)	Reference
bla$_{VIM}$	F	5`… ATTGGTCTATTTGACCGCGTC	780	29, 31 and 32
	R	5`… TGCTACTCAACGACTGAGCG		
bla$_{IMP}$	F	5`… CATGGTTTGGTGGTTCTTGT	488	29, 31 and 32
	R	5`… ATAATTTGGCGGACTTTGGC		
bla$_{KPC}$	F	5`… CGTTGACGCCCAATCC	390	29, 31 and 32
	R	5`… ACCGCTGGCAGCTGG		
bla$_{OXA-48}$	F	5`…ATGCGTGTATTAGCCTTATCGGC	770	29, 31 and 32
	R	5`…ACTTCTTTTGTGATGGCTTGGCGCA		
bla$_{NDM-1}$	F	5`… GGTTTGGCGATCTGGTTTTC	621	30
	R	5`… CGGAATGGCTCATCACGATC		
mcr-1	F	5`… CGGTCAGTCCGTTTGTTC	309	28
	R	5`… CTTGGTCGGTCTGTAGGG		
ERIC-2		5`… AAGTAAGTGACTGGGGTGAGCG		33

F: Forward, R: Reverse

standard microbiological procedures.[21] All isolates were cultured at 37°C in Luria Bertani medium (LB broth; tryptone 1% w/v, yeast extract 0.5% w/v and NaCl 1.0% w/v), otherwise specified, and stored in 50% glycerol/LB broth at -80°C. During the culture and sensitivity testing, the isolates were cultured on modified Muller-Hinton agar (MHA) supplemented with all antimicrobials, except for colistin as described before.[22] For colistin, to improve its diffusion, the modified MHA containing 30% agar was used.

Antimicrobial sensitivity testing

Antimicrobial susceptibility tests were performed on the isolates against several antimicrobial classes (Oxoid, UK) including β-lactams (sulbactam, cefepime, cefoperazone, ceftazidime, carbenicillin, ceftriaxone, cefotaxime, ampicillin, meropenem and imipenem), quinolones (levofloxacin, ciprofloxacin, norfloxacin and nalidixic Acid), aminoglycosides (amikacin, gentamicin and tobramycin), chloramphenicol and colistin by using disc diffusion method.[23] Antimicrobial susceptibility was established using the clinical and laboratory standards institute (CLSI) 2019 and European committee on antimicrobial susceptibility testing (EUCAST) recommendations.[6,22,24] As a control, *Escherichia coli ATCC 25922* standard strain was used during these experiments.

Carbapenemase phenotypic examination

Modified Hodge test (MHT) was carried out to test carbapenemase production as described before.[25] A commercial disc containing 10 µg of imipenem was placed in the middle of a MHA plate that had been inoculated with *E. coli ATCC 25922*. The tested *P. aeruginosa* isolates were considered β-lactamase positive if they enabled *E. coli ATCC 25922* strain to resist the imipenem giving a cloverleaf-like indentation. During these experiments, *K. pneumoniae ATCC BAA-1705* and *ATCC BAA-1706* strains were used as positive and negative controls, respectively.

EDTA synergistic test was used to evaluate the MBLs activity as indicated before.[26] This test involves challenging the tested isolate with one disc containing anhydrous EDTA (292 µg) (Sigma Chemicals in St. Louis, MO) and two imipenem discs (10 µg each). The discs are spaced 25 mm apart in the MHA plate. Positive MBL production was demonstrated by an increase in the inhibition zone width of more than 4 mm around the imipenem-EDTA disc in comparison to the imipenem disc alone. As a control, *E. coli ATCC 25922* standard strain was used during these experiments.

Furthermore, KPC enzyme synthesis evaluation was established by the boronic acid test as specified before.[27] Antimicrobial discs (cefepime, meropenem or imipenem) and a KPC

Table 2. Distribution of carbapenem and colistin resistance genes in *P. aeruginosa* tested isolates and their phenotypic detection

Isolate code	Source	Sex	Ward	Infection	bla_{VIM}	bla_{KPC}	bla_{NDM-1}	bla_{IMP}	bla_{OXA-48}	*mcr-1*	MHT	EDTA synergistic test	Boronic acid test
U5	Urine	F	UNC	UTI	+	+	-	-	-	-	-	+	+
U6	Urine	F	MUH	UTI	+	+	-	+	-	-	-	+	+
U7	Urine	F	CUH	UTI	+	+	+	+	-	-	+	+	+
U8	Urine	F	MUH	UTI	+	+	-	-	-	-	-	+	+
S9	Sputum	F	MUH	RTI	+	+	+	+	-	-	+	+	+
U10	Urine	F	UNC	UTI	+	+	-	+	-	-	-	+	+
U11	Urine	M	MUH	UTI	+	+	+	+	-	-	+	+	+
U12	Urine	M	UNC	UTI	+	+	-	+	-	-	+	+	+
U13	Urine	M	ICU	UTI	+	-	-	-	-	-	-	+	-
U14	Urine	M	MUH	UTI	+	-	+	-	-	-	-	+	-
U23	Urine	F	MUH	UTI	+	+	-	+	-	-	-	+	+
U24	Urine	F	UNC	UTI	+	+	-	+	-	-	+	+	+
U25	Urine	M	MUH	UTI	+	+	-	+	-	+	-	+	+
U26	Urine	F	CUH	UTI	+	+	-	-	-	-	+	+	+
U35	Urine	F	MUH	UTI	+	-	+	-	-	+	-	+	-
U36	Urine	F	UNC	UTI	+	+	-	-	-	-	-	+	+
U40	Urine	F	CUH	UTI	+	+	-	-	+	-	+	+	+
W41	wounds	F	ICU	WI	+	+	-	-	-	-	+	+	+
W42	wounds	F	ICU	WI	+	-	-	+	-	-	+	+	-
W43	wounds	F	ICU	WI	+	-	-	-	-	-	+	+	-
W44	wounds	F	ICU	WI	+	-	+	-	-	-	+	+	-
W45	wounds	F	CUH	WI	-	+	-	-	-	+	-	-	+
W46	wounds	M	ICU	WI	+	-	-	-	-	-	+	+	-
W50	wounds	M	BCC	WI	-	+	-	-	+	-	-	-	+
W51	wounds	M	BCC	WI	-	+	-	+	-	-	+	+	+
W52	wounds	M	BCC	WI	-	-	-	+	-	-	-	+	-
W54	wounds	M	MUH	WI	-	-	-	+	-	-	+	+	-
W55	wounds	M	MUH	WI	+	+	-	-	-	-	+	+	+
B56	Burns	M	BCC	BI	+	+	+	-	+	+	-	+	+
B62	Burns	M	BCC	BI	-	-	-	-	+	-	+	-	-
B63	Burns	M	BCC	BI	-	-	+	-	+	-	+	+	-
B65	Burns	M	BCC	BI	-	+	+	-	+	+	-	+	+
S66	Sputum	M	CH	RTI	-	-	-	-	+	-	+	-	-
S68	Sputum	M	CH	RTI	+	-	+	-	-	-	+	+	-
S69	Sputum	F	MIH	RTI	+	-	+	-	-	-	+	+	-
S71	Sputum	M	MIH	RTI	+	-	-	-	-	-	+	+	-
B73	Burns	F	BCC	BI	-	+	+	+	-	-	-	+	+
W74	wounds	F	BCC	WI	+	+	-	-	+	-	+	+	+
B75	Burns	M	BCC	BI	+	+	-	-	-	+	+	+	+
U76	Urine	F	UNC	UTI	+	+	-	+	+	-	+	+	+
U78	Urine	F	CUH	UTI	+	+	-	-	+	-	-	+	+
U80	Urine	F	MUH	UTI	+	+	-	-	+	+	+	+	+
EY81	Eye	M	OC	EI	+	+	-	-	-	+	+	+	+
S82	Sputum	M	MUH	RTI	+	+	-	-	-	-	-	+	+
EY83	Eye	M	OC	EI	+	+	-	-	-	-	+	+	+
B84	Burns	M	BCC	BI	+	+	+	-	+	+	-	+	+
U85	Urine	M	MUH	UTI	+	+	+	-	+	-	-	+	+
U86	Urine	F	MUH	UTI	+	+	-	-	-	+	+	+	+

Table 2. Cont...

Isolate code	Source	Sex	Ward	Infection	*bla*$_{VIM}$	*bla*$_{KPC}$	*bla*$_{NDM-1}$	*bla*$_{IMP}$	*bla*$_{OXA-48}$	*mcr-1*	MHT	EDTA synergistic test	Boronic acid test
E87	Ear	F	MUH	OM	+	+	+	-	+	-	-	+	+
E90	Ear	F	CUH	OM	+	+	+	-	+	-	-	+	+
B95	Burns	M	BCC	BI	-	+	-	-	-	-	-	-	+
W96	wounds	F	MUH	WI	-	-	+	-	-	-	-	+	-
E97	Ear	F	MUH	OM	+	-	-	-	-	-	+	+	-
U98	Urine	M	MUH	UTI	+	-	-	+	-	-	+	+	-
E99	Ear	M	CUH	OM	-	+	-	-	-	-	+	-	+
B100	Burns	M	BCC	BI	+	+	-	-	-	-	-	+	+

B: Burns, BCC: Burns and cosmetics center, BI: Burn infection, CH: Chest hospital, CUH: Cairo university hospital, E: Ear swabs, EI: Eye infection, EY: Eye, F: Female, ICU: Infection control unit, M: Male, MHT: Modified Hodge test, MIH: Mansoura international hospital, MUH: Mansoura university hospital, OC: Ocular center, OM: Otitis media, RTI: Respiratory tract infection, S: Sputum, U: Urine, UNC: Urology and nephrology center, UTI: Urinary tract infection, W: Wound, WI: Wound infection

inhibitor (400 µg benzene boronic acid; Sigma-Aldrich, Steinheim, Germany) were employed in this assay. The tests were carried out by inoculating a MHA plate with the tested isolate in the presence of antimicrobial discs with or without boronic acid. Antimicrobial-boronic acid disk is compared to the antibiotic disc alone, and KPC enzyme production is considered positive if the diameter of the inhibitory zone around the former increases by 5 mm or more. The standard strain *E. coli ATCC 25922* was used in this test to establish quality control.

Polymerase chain reaction (PCR)

The QIA amp® DNA miniprep kit (Qiagen, Germany) was utilized to extract plasmid DNA from *P. aeruginosa* isolates. Screening for the existence of β-lactamases genes involving *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM-1}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$ and colistin resistance gene (*mcr-1*) was established by PCR using the primer sets listed in Table 1.[28-32] The PCR reactions were established in the volume of 25 µL. Each PCR reaction consisted of 3 µL of template DNA, 1 µL forward primer (10 µM), 1 µL of reverse primer (10 µM), 12.5 µL Dream Taq PCR master mix 2x (Fermentas, USA) and 7.5 µL nuclease free water. Tubes with no template DNA were used as a negative control. The PCR cycling was established as follows; primary denaturation for 5 min at 95°C, followed by 35 cycles of (denaturation for 30 s at 95°C, annealing for 30 s at 52°C for *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM-1}$ and *mcr*-1, while at 48°C and 58°C for *bla*$_{KPC}$ and *bla*$_{OXA-48}$, respectively and extension for 1 min at 72°C) and last extension for 5 min at 72°C.

Results of PCR were inspected using gel electrophoresis, where the products were run on 1.2% agarose gel and visualized utilizing ethidium bromide (MP biomedicals, France). The size of the obtained amplicons was matched to DNA ladder (Thermo Scientific, UK). The tested gene was considered positive if a single sharp band of its expected size appeared beside the matched band of the DNA ladder reflecting its size.

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

The clonal relatedness of the *P. aeruginosa* isolates was done using ERIC-PCR. The banding profile was generated using the oligonucleotide primer ERIC-2 (Table 1)[33] according to the method previously described.[34] The resultant patterns attained from ERIC-PCR were construed using the software Past® (version 4.01).[35] The similarities among the fingerprints were calculated based on Pearson correlation (optimization, 1%; position tolerance, 1%). By using the UPGMA algorithm, the fingerprints were sorted based on their similarity to generate their corresponding dendrograms.[36] Isolates with a resemblance of more than 85% were deemed clonal.

Table 3. Antimicrobial susceptibility patterns of *P. aeruginosa* tested isolates

Isolate code	Antimicrobial class																		
	β-lactams										Quinolones				Amino-glycosides			Lipo-peptide	
	SAM	FEP	CFP	CAZ	CAR	CRO	CTX	AMP	MEM	IPM	LEV	CIP	NOR	NA	AK	TOB	CN	CT	C
U5	R	S	R	S	S	R	R	R	S	R	S	S	S	R	S	S	S	S	S
U6	R	R	R	I	S	R	R	R	R	S	S	S	S	R	S	S	S	S	R
U7	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	S	R
U8	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
S9	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
U10	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R
U11	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
U12	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
U13	R	R	R	R	R	R	R	R	R	R	R	I	I	R	S	R	R	S	R
U14	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	S	R
U23	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
U24	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	R	S	R
U25	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
U26	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
U35	R	R	R	R	R	R	R	R	R	S	R	R	R	R	I	R	R	R	R
U36	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	I	R	S	R
U40	R	R	R	R	R	R	R	R	R	R	S	R	R	R	I	R	R	S	R
W41	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	S	R
W42	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	S	R
W43	R	R	R	R	R	R	R	R	R	I	R	R	R	R	S	R	R	S	R
W44	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	S	R	S	R
W45	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
W46	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	I	R	S	R
W50	R	R	R	R	R	R	R	R	S	R	S	S	S	R	R	R	R	S	R
W51	R	I	R	R	R	R	R	R	R	S	R	R	R	R	S	S	S	S	R
W52	R	S	R	R	S	I	R	R	R	S	S	S	S	R	S	S	S	S	S
W54	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
W55	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	S	R
B56	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
B62	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R
B63	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	R	S	R
B65	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	R	R	R
S66	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	S	R
S68	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	I	R	S	R
S69	R	R	R	R	R	R	R	R	R	R	S	S	S	R	I	R	S	S	R
S71	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	S	R	S	R
B73	R	R	R	R	R	R	R	R	R	R	I	S	S	R	S	R	R	S	I
W74	R	R	R	R	R	R	R	R	R	R	R	S	S	R	S	R	R	S	I
B75	R	R	R	R	R	R	R	R	R	R	I	S	S	R	S	R	R	R	I
U76	R	R	R	R	R	R	R	R	R	R	R	S	S	R	S	R	R	S	I
U78	R	I	R	R	R	R	R	R	R	R	R	I	S	R	I	R	R	S	I
U80	R	R	R	R	R	R	R	R	R	R	I	I	S	R	S	R	R	R	I
EY81	R	I	R	R	R	R	R	R	R	R	I	S	S	R	S	R	R	R	I
S82	R	R	R	R	R	R	R	R	R	R	I	I	S	R	S	R	S	S	I
EY83	R	R	R	R	R	R	R	R	R	R	I	S	S	R	S	R	S	S	I
B84	R	R	R	R	R	R	R	R	R	R	R	I	S	R	S	R	S	R	I
U85	R	R	R	R	R	R	R	R	R	R	R	I	S	R	R	R	S	S	I
U86	R	R	R	R	R	R	R	R	R	R	I	S	S	R	R	R	S	R	I
E87	R	S	R	R	R	I	R	R	R	I	R	R	R	R	S	R	R	S	R

Table 3. Cont...

Isolate code	Antimicrobial class																		
	β-lactams										Quinolones				Amino-glycosides			Lipo-peptide	
	SAM	FEP	CFP	CAZ	CAR	CRO	CTX	AMP	MEM	IPM	LEV	CIP	NOR	NA	AK	TOB	CN	CT	C
E90	R	S	R	I	S	R	R	R	R	I	S	S	S	R	S	S	S	S	R
B95	R	R	R	R	R	R	R	R	R	R	S	I	S	R	S	R	R	S	I
W96	R	R	R	R	R	R	R	R	R	R	I	S	S	R	S	R	R	S	I
E97	R	R	R	R	I	I	R	R	R	S	R	R	S	R	R	R	R	S	I
U98	R	R	R	R	R	R	R	R	R	R	I	I	S	R	S	R	R	S	I
E99	R	R	R	R	R	R	R	R	R	R	S	S	S	R	S	R	R	S	I
B100	R	R	R	R	R	R	R	R	R	R	I	S	S	R	S	R	R	S	I
Total	100%	87.50%	100%	94.60%	91.10%	94.60%	100%	100%	96.40%	75%	55.30%	46.40%	44.60%	100%	44.60%	80.35%	80.35%	17.80%	62.50%

AK: Amikacin, AMP: Ampicillin, B: Burns, C: Chloramphenicol, CAR: Carbenicillin, CAZ: Ceftazidime, CFP: Cefoperazone, CIP: Ciprofloxacin, CN: Gentamicin, CRO: Ceftriaxone, CT: Colistin, CTX: Cefotaxime, E: Ear swabs, EY: Eye, FEP: Cefepime, IPM: Imipenem, LEV: Levofloxacin, MEM: Meropenem, NA: Nalidixic Acid, NOR: Norfloxacin, S: Sputum, SAM: Sulbactam, TOB: Tobramycin, U: Urine, W: Wound

RESULTS

Clinical features and antimicrobial sensitivity patterns of the tested isolates

All tested *P. aeruginosa* were carbapenemase-producing isolates and harboured carbapenemase genes. According to the clinical source as shown in Table 2, 22 isolates (39.28%) were gathered from urine, 13 isolates (23.21%) from wounds, 9 isolates (16.1%) from burns, 6 isolates (10.71%) from sputum, 4 isolates (7.14%) from ear swabs and 2 isolates (3.57%) from eye infections. In a 1:1 ratio, samples were drawn from both males and females. Regarding the antimicrobial susceptibility patterns of the tested isolates as shown in Table 3, the highest resistance percentage was observed with sulbactam/amoxicillin, cefoperazone, cefotaxime, ampicillin and nalidixic acid, where 100% of the isolates were resistant. Moreover, the resistance percentage to ceftazidime and ceftriaxone was 94.6%, followed by carbenicillin (91.1%) and both tobramycin and gentamicin (80.35%). The resistance percentages to meropenem and imipenem were 96.4% and 75%, respectively. On the other hand, colistin was shown to have the lowest resistance rate (17.8%), while amikacin and norfloxacin resistance was (44.6%). In addition, the resistance percentages to levofloxacin, ciprofloxacin and chloramphenicol were 55.3%, 46.4% and 62.5%, respectively. The examined isolates U25 and B56 showed complete resistance to all antimicrobials used in this study.

Phenotypic and genotypic detection of carbapenemases and MCR-1 among the tested isolates

According to Table 2, 31 isolates (55.35%) were MHT positive. Furthermore, the EDTA synergistic test was positive in 50 isolates (89.23%). In addition, the boronic acid test gave positive findings with 38 isolates (67.85%).

PCR detection of genes encoding carbapenemase and MCR-1 confirmed the phenotypic outcomes as shown in Table 2, bla_{VIM}, bla_{KPC}, bla_{NDM-1}, bla_{IMP} and bla_{OXA-48} genes were detected in 43 (76.7%), 38 (67.7%), 17 (30.3%), 16 (28.5%) and 15 (26.7%) isolates, respectively. bla_{VIM} was the most frequent gene in the tested isolates. On the other hand, bla_{OXA-48} was the least common gene. In addition, 43 isolates (76.78%) harboured more than one carbapenemase gene as shown in Table 4, while 13 (23.2%) isolates harboured only one gene as shown in Table 5. Regarding *mcr-1* gene, it was detected in 10 *P. aeruginosa* carbapenemase-producing isolates, which were collected from urine (4 specimens), wound (1 specimen), burns (4 specimens) and eye infection (1 specimen) (Tables 2, 4 and 5).

Genetic relatedness of the *P. aeruginosa* tested isolates

ERIC-PCR analysis of the isolates, as shown in Figure, revealed that they are unrelated to each other, except for the isolates (EY83 and B84), (S66 and S68) and (W74 and B75), which have more than 85% similarity and were considered clonal.

DISCUSSION

P. aeruginosa is one of the most prevalent Gram-negative opportunistic bacteria that can result in nosocomial infections. The scenario becomes worse if the infections are linked to MDR pathogens, which limits the treatment options [37]. Indeed, the resistance of Gram-negative pathogens, including *P. aeruginosa*, to carbapenems is a global health concern.[10]

This study found that each isolate of the collected *P. aeruginosa* was at least resistant to one carbapenem (imipenem or meropenem). Moreover, 50 isolates (89.23%) were MBL producers. Previous reports showed similar results, where a study indicated that 12 *P. aeruginosa* isolates of 80 (26.25%) were carbapenemase producers.[10] According to another study, 14 *P. aeruginosa* isolates of 114 (12.2%) were carbapenem resistant. Thirteen isolates of these 14 isolates (11.4%) exhibited the MBL phenotype.[11] Another two studies established in Brazil and Korea demonstrated that 43.9% and 92.7% of *P. aeruginosa* isolates were resistant to carbapenem, respectively.[20,38] In addition, a study that was carried out in Canada depicted that 228 patients were infected with imipenem resistant *P. aeruginosa*. This study demonstrated that 98 isolates were MBL producers.[39] In addition, a study conducted in Iran indicated that 110 isolates out of 122 (90 %) were imipenem resistant and MBL producers.[40]

Indeed, MHT is a confirmatory phenotypic test for the inspection of *P. aeruginosa* carbapenemase synthesis. However, it should be noted that the sensitivity and specificity of MHT for detecting carbapenemase synthesis are debatable. Although all the tested isolates generated carbapenemases, 31 isolates (55.3%) were MHT

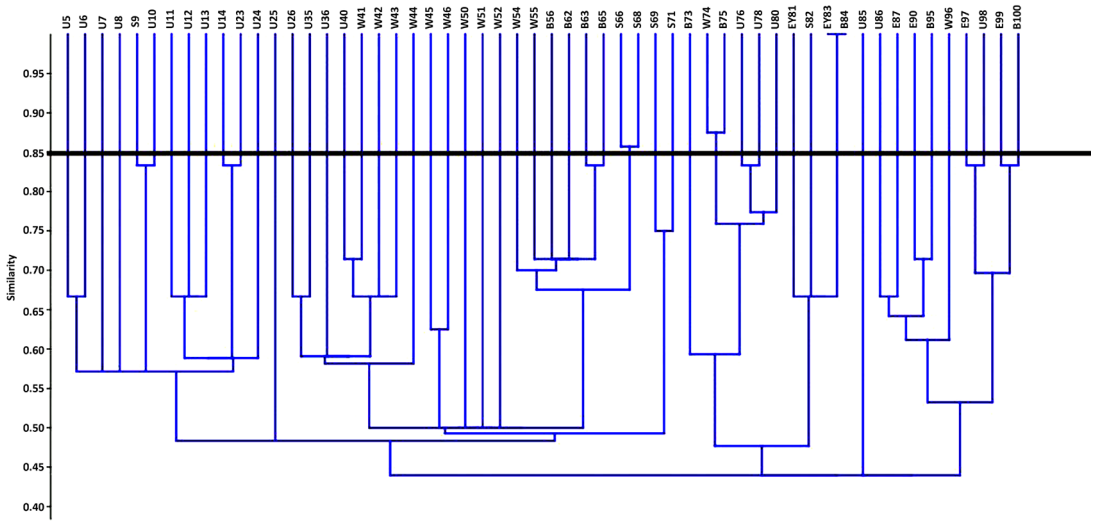

Figure. Dendrogram of ERIC-PCR exhibiting the clonal relatedness of *P. aeruginosa* isolates. The resulting patterns obtained from ERIC-PCR were interpreted using the software Past® (version 4.01). The similarities between the fingerprints were calculated based on Pearson correlation (optimization, 1%; position tolerance, 1%), and the fingerprints were grouped according to their similarities by using UPGMA algorithm to generate their corresponding dendrograms. Isolates with > 85% similarity were considered clonal. ERIC-PCR, enterobacterial repetitive intergenic consensus polymerase chain reaction

positive and the remaining 25 isolates (44.6%) showed negative results. Several investigations have also indicated that the MHT may produce false positive or negative results during the detection of carbapenemase synthesis in Gram-negative pathogens.[41-43] Unlike MHT, EDTA synergistic test used to inspect MBL synthesis, including VIM, NDM-1 and IMP, was shown to be significantly receptive, because all MBL *P. aeruginosa* producing isolates (50 isolates) were EDTA synergistic test positive. Furthermore, the boronic acid test was extremely responsive for detecting KPC enzyme synthesis, because of giving positive results with all KPC-positive *P. aeruginosa* isolates (38 isolates). Therefore, our findings suggest that the EDTA synergistic test and the boronic acid test can be routinely used in microbiology laboratories to check MBL and KPC producing *P. aeruginosa* isolates.

Former reports stated that the bla_{VIM} gene is the most common produced gene in carbapenem-resistant *P. aeruginosa.*[10,11,13,14] This is in accordance with this study, where bla_{VIM} was harboured by 46 *P. aeruginosa* isolates (76.78%) as the most common MBL. In a study conducted in Egypt, bla_{VIM} gene was found in 8 (57%) isolates out of 14 *P. aeruginosa* imipenem resistant isolates [11]. Moreover, this gene was found in 19 (55.88%) isolates out of 34 carbapenem-resistant *P. aeruginosa* isolates in another report.[10] In two Iranian studies,[40] and[44] the prevalence rates of the bla_{VIM} gene were reported to be 1.6% and 55%, respectively. Another study established in Canada indicated that 90 (39.47%) isolates out of 228 imipenem resistant *P. aeruginosa* isolates harboured bla_{VIM} gene. Moreover, during the year 2003, a nosocomial outbreak was caused by a cluster of bla_{VIM} producing strains.[39] Another study in Poland depicted a higher incidence of *P. aeruginosa* strains harbouring the bla_{VIM} gene (68%).[45]

In this study, bla_{IMP} gene was found in 16 *P. aeruginosa* isolates (28.57%). In Egypt, a study demonstrated that 5 isolates out of 14 imipenem

Table 4. *P. aeruginosa* isolates that harbour more than one carbapenem-resistant gene accompanied with the colistin resistant gene, *mcr-1*

Carbapenem resistance genes and *mcr-1*	Isolate codes	Total isolates (56 isolates)
bla_{VIM} + bla_{KPC} + bla_{NDM-1}+ bla_{IMP}	U7+S9+U11	3 (5.35%)
bla_{VIM} + bla_{KPC} + bla_{NDM-1}+ bla_{OXA-48}	E87+E90+U85	3 (5.35%)
bla_{VIM} + bla_{KPC}+ bla_{NDM-1}+ bla_{OXA-48} + *mcr-1*	B56+B84	2 (3.57%)
bla_{VIM} + bla_{KPC} + bla_{IMP} + bla_{OXA-48}	U76	1 (1.78%)
bla_{VIM} + bla_{KPC} + bla_{IMP}	U6+U10+U12+U23+U24	5 (8.92%)
bla_{VIM} + bla_{KPC} + bla_{IMP} + *mcr-1*	U25	1 (1.78%)
bla_{VIM} + bla_{KPC} + bla_{OXA-48}	W74+U78+U40	3 (5.35%)
bla_{VIM} + bla_{KPC} + bla_{OXA-48} + *mcr-1*	U80	1 (1.78%)
bla$_{KPC}$ + bla_{NDM-1}+ bla_{OXA-48} + *mcr-1*	B65	1 (1.78%)
bla$_{KPC}$ + bla_{NDM-1}+ bla_{IMP}	B73	1 (1.78%)
bla_{VIM} + bla_{KPC}	U5+U8+U26+U36+W41+ W55+S82+EY83+B100	9 (16.1%)
bla_{VIM} + bla_{KPC} + *mcr-1*	B75+EY81+U86	3 (5.35%)
bla_{VIM} + bla_{NDM-1}	U14+W44+S68+S69	4 (7.1%)
bla_{VIM} + bla_{NDM-1} + *mcr-1*	U35	1 (1.78%)
bla_{VIM} + bla_{IMP}	W42+U98	2 (3.57%)
bla_{KPC} + bla_{IMP}	W51	1 (1.78%)
bla_{KPC} + bla_{OXA-48}	W50	1 (1.78%)
bla_{NDM-1}+ bla_{OXA-48}	B63	1 (1.78%)
Total		43 (76.7%)

Table 5. *P. aeruginosa* isolates that harbour only one carbapenem-resistant gene accompanied with the colistin resistant gene, *mcr-1*

Carbapenem resistance gene and *mcr-1*	Isolate codes	Total isolates (56 isolates)
bla_{VIM}	W46+E97+U13+ W43+S71	5 (8.92%)
bla_{KPC}	B95+ E99	2 (3.57%)
bla_{KPC} + *mcr-1*	W45	1 (1.78%)
bla_{NDM-1}	W96	1 (1.78%)
bla_{IMP}	W52+W54	2 (3.57%)
bla_{OXA-48}	B62+S66	2 (3.57%)
Total		13 (23.2%)

resistant *P. aeruginosa* isolates (35%) harboured bla_{IMP} gene.[11] Other studies found lower incidence rates of bla_{IMP} including 1.75% in Canada,[39] and 3% in Iran.[44] Another study in Iran, on the other hand, depicted a greater prevalence of bla_{IMP} (55%) through the tested isolates of *P. aeruginosa.*[40]

In Egypt, the first report of *P. aeruginosa* harbouring bla_{NDM-1} was published in 2014,[14] where the authors showed that 2 *P. aeruginosa* isolates out of 33 (6%) were carbapenem-resistant and harboured bla_{NDM-1}. Moreover, another report showed a greater incidence of *P. aeruginosa* isolates that are resistant to carbapenem carrying bla_{NDM-1}.[13] According to this study, bla_{NDM-1} gene was found in 17 *P. aeruginosa* carbapenems and carrying isolates (30.4%) and this percentage is high in relation to the previous reports.

Regarding bla_{KPC}, in Egypt, a study demonstrated that this gene was found in 1 isolate (2.9%) out of 34 *P. aeruginosa* carbapenemase-producing isolates, while bla_{OXA-48} gene was not found in all 34 carbapenemase-producing isolates.[10] In the current study, bla_{KPC} and bla_{OXA-48} occurred in 38 (67.85%) and 15 (26.78%) isolates, respectively. These incidence percentages of both genes are much higher than the previous reports. Another study conducted in Iran indicated that bla_{KPC} presented in 13% of 108 *P. aeruginosa* isolates,[41] while in Puerto Rico, 99 (4.1%) out of 2415 *P. aeruginosa* isolates harboured bla_{KPC} gene [46]. Moreover, a study in Sudan reported that 60% of *P. aeruginosa* isolates carried bla_{OXA-48} gene.[47]

According to this study, 43 (76.78%) *P. aeruginosa* isolates carried more than one carbapenem resistance gene. Compared to another report in Egypt, this percentage is very high. Previous report showed that 1 isolate out of 34 *P. aeruginosa* carbapenemase-producing isolates harboured both bla_{VIM} and bla_{KPC}.[10] Another study depicted that only 4 (28.5%) isolates out of 14 *P. aeruginosa* carbapenemase-producing isolates carried both bla_{VIM} and bla_{IMP}.[11] This gives an indication for the prevalence of carbapenem resistance genes that increased and became a serious problem in Egypt.

According to the data of this study, the lowest percentage of resistance among the tested *P. aeruginosa* isolates was recorded for colistin (10%). Given the fact that colistin has the potential to be nephrotoxic, it should only be used as a last choice for serious infections that cannot be cured with antimicrobial combinations.[48] In this study, all *P. aeruginosa* colistin resistant isolates harboured *mcr-1* gene. These data revealed high colistin resistance percentage compared to previous studies that showed complete sensitivity to colistin [10] and another study that demonstrated that only 1 isolate from 66 *P. aeruginosa* isolates was resistant to colistin.[28]

In the present study, the homology analysis (using ERIC-PCR) revealed that plasmid-mediated carbapenemases and colistin resistance genes could be easily transmitted across the *P. aeruginosa* isolates. Most of the tested isolates were unrelated to each other; however, they all included one or more carbapenemase gene. According to these findings, it can be concluded that *P. aeruginosa* isolates can easily share plasmids that encode antimicrobial resistance markers. These findings support earlier studies that described the horizontal spread of plasmids encoding carbapenemases as well as MCR-1 among Gram-negative bacteria.[4,10,11,13,14,20,38,41,47,49]

Overall, one of the biggest threats to the healthcare systems globally is the appearance of MDR pathogens. This study demonstrated the presence of MDR *P. aeruginosa* isolates that are resistant to both carbapenems and colistin as well as other antimicrobials. Early inspection of MDR *P. aeruginosa* isolates with any diminished susceptibility to the carbapenems as well as colistin is essential for the choice of the most proper antimicrobial treatment and the application of effective infection management

protocols. Increasing the health awareness and the appropriate use of antimicrobials, particularly carbapenems and cephalosporins, as well as the minimalistic use of colistin may help to prevent the emergence of such resistance patterns.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

FS, TEB and AE designed the study. KMA collected the bacterial isolates. KMA and AE performed experiments. KMA and AE analyzed the results and established the tables and figures. KMA and AE wrote the manuscript. FS and TEB revised the manuscript. All authors read and approved the final manuscript for publication.

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None.

DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was approved by Research Ethics Committee of the Faculty of Pharmacy, Tanta University, Egypt, (Research Ethics Committee Code: TP / RE /12-21-M-002).

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