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Original Article

Molecular characterization of resistance mechanisms in *Pseudomonas aeruginosa* isolates resistant to carbapenems

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Abstract

Introduction: Emergence of carbapenem resistance in *Pseudomonas aeruginosa* increases the therapeutic dilemma. In this study, we investigated various mechanisms involved in the resistance of *P. aeruginosa* clinical isolates to carbapenems.

Methodology: *P. aeruginosa* isolates were isolated from different clinical samples. The antimicrobial susceptibility was evaluated by disc diffusion method. Carbapenemases were detected among carbapenem resistant isolates. Expression level of mexB and oprD was determined by real-time PCR. Molecular relatedness among isolates was detected based on pulsed-field gel electrophoresis (PFGE).

Results: Ninety *P. aeruginosa* isolates were purified from clinical specimens. High levels of resistance to imipenem and meropenem were detected in 16 isolates. PCR analysis of carbapenemases indicated the prevalence of Verona integron-encoded metallo-beta-lactamase (VIM); four isolates produced only VIM enzymes (VIM-1 or VIM-2), while the remaining twelve co-produced both VIM-1 or VIM-2 and NDM enzymes.

Additionally, real-time PCR analysis elucidated high expression levels of *mexB* in seven of the carbapenem resistant isolates and low expression of *oprD* in seven isolates.

The identified carbapenem-resistant isolates were clustered into eleven PFGE profiles where clusters E1 and E2 involved isolates exhibiting multiple carbapenemase genes (bla_{NDM-1}, bla_{VIM-1} and bla_{VIM-2}).

Conclusion: Various mechanisms underlying carbapenem resistance have been detected in our *P. aeruginosa* cohort of isolates. Emergence of *P. aeruginosa* as a reservoir of multiple carbapenemases is increasing over time limiting the treatment options to this serious infection. This increases the urgency for infection control practices to reduce the incidence of this infection.

Key words: carbapenem resistance; *Pseudomonas aeruginosa*; carbapenemases; efflux pump; outer membrane protein.

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Introduction

Pseudomonas aeruginosa is one of the most important opportunistic pathogens that has been associated with community and hospital-acquired infections such as respiratory tract infections, burns, wounds, otitis media and nosocomial infections [1]. The frequency of infections caused by *P. aeruginosa* is increasing and multidrug-resistant (MDR) isolates are emerging in hospitalized patients [2].

Carbapenems have been kept as a last resort therapy for the control of MDR *P. aeruginosa* infection. However, carbapenem-resistant *P. aeruginosa* could be resistant to other classes of antimicrobial agents and such infections are associated with limited therapeutic options and high rates of mortality and morbidity especially in hospitalized and immunocompromised patients [3-4].

Various mechanisms contribute to the development of carbapenem-resistant *P. aeruginosa* particularly through the acquisition of resistant genes encoding carbapenem-hydrolyzing enzymes [5-6]. A wide range of metallo-β-lactamases (MBLs) (class B β-lactamases) such as IMP, VIM, NDM-1 and GIM-1 have been reported in *P. aeruginosa*. These enzymes play a major role in the resistance of *P. aeruginosa* to carbapenems [7]. In addition, class A β-lactamase *Klebsiella pneumoniae* carbapenemases (KPC) carries the extended spectrum KPC-1 enzyme, which was first detected in an outbreak in North Carolina [8], has subsequently been identified in *P. aeruginosa* isolates [9].

Furthermore, *P. aeruginosa* isolates may exhibit multidrug efflux machinery, mainly MexAB-OprM and MexXY-OprM. Efflux of carbapenems represents a principal resistance mechanism in *P. aeruginosa* [10].

The transport of carbapenems across the lipid bilayer membranes occurs through protein channels, porins, especially OprD [11]. Lack of OprD participates in the resistance of *P. aeruginosa* isolates to carbapenems [12-13]. The aim of this study is to examine the coexistence of different genes encoding carbapenemases in *P. aeruginosa* clinical isolates. Also, the expression level of porins (OprD) and efflux machinery were evaluated as contributing mechanisms of carbapenem resistance in these isolates. Moreover, the genotypic profile of *P. aeruginosa* clinical isolates was determined using pulsed-field gel electrophoresis (PFGE). This can shed more light on promising targets for developing strategies to defend the dissemination of carbapenem resistance, propose infection control regimens and assign suitable treatment for such infection.

Methodology

P. aeruginosa clinical isolates

Gram-negative bacilli were recovered from various sources, including sputum, urine and pus exudates between May 2015 and September 2015. Ninety *P. aeruginosa* isolates were identified according to standard laboratory methods [14]. *P. aeruginosa* isolates were collected during outpatient treatment at University Hospital (255 bed), Chest Hospital (214 bed), Urology and Nephrology Center (270 bed), International Hospital (400 bed) and from inpatient admitted Burns and Cosmetics Center (48 bed) at this period. Luria-Bertani medium was used for propagation of bacterial growth. Glycerol stocks (30%) were prepared and stored at -80 ºC. The methodology applied in this research follow the ethical guidelines of "The Research Ethics Committee, Faculty of Pharmacy, Mansoura University".

Assay of antimicrobial susceptibility

The susceptibility of the purified isolates to different types of antimicrobial agents was evaluated using disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015) [15]. The tested antimicrobial agents include ceftazidime (CAZ), imipenem (IPM), meropenem (MEM), amikacin (AK), gentamicin (CN), levofloxacin (LEV) (Oxoid, Basingstoke, UK). The susceptibility profiles were interpreted using the Clinical and Laboratory Standards outlines [15].

Determination of the MICs

Minimum inhibitory concentrations (MICs) of imipenem and meropenem against our isolates were determined by microtitre plate broth dilution method. Two-fold serial dilutions of imipenem or meropenem were prepared in Mueller-Hinton broth as follows 2048, 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 µg per mL. The dilutions were inoculated with 0.5×10^5 colony-forming units per mL of the tested isolates and the plates were incubated for 18 hours at 37 °C [15]. Bacterial growth was assessed and the MIC for each isolate was quantified as the lowest concentration of the antibiotic that prevents visible bacterial growth.

Modified Hodge Test (MHT)

The presence of carbapenemases in *P. aeruginosa* isolates was primary detected using Modified Hodge test. The diluted culture of *Escherichia coli* ATCC 25922 (0.5 McFarland standard) was swabbed on the surface of Mueller-Hinton agar plates in three different directions. Meropenem disk (10 µg) (Oxoid, Basingstoke, UK) was placed at the center of each plate. The tested isolates were streaked as a thin line from the edge of the meropenem disk to the edge of the plate [16]. Bacterial growth was allowed for 18 hours at 37 °C. Indentation in the inhibition zone of *E. coli* or clove growth of *E. coli* around the meropenem disk revealed a positive MHT [15].

PCR of carbapenemases encoding genes

The carbapenemases encoding genes; bla_{NDM-1}, bla_{VIM-1}, bla_{VIM-2}, bla_{OXA}, bla_{IMP}, bla_{KPC} were screened by PCR using 0.5 U FlexiTaq DNA polymerase (Promega, Madison, USA), 5 × GoTaq Flexi buffer, 0.5 mM of each primer (Table 1), 0.1 mM of each dNTP, 0.5 mM $MgCl_2$ solution and H_2O up to 20 µL. The thermocycling conditions started with initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing for 30 seconds at temperatures specific to each primer pair as mentioned in Table 1 and extension at 72°C for 30 seconds and the reaction ended with a final extension at 72 °C for 5 minutes.

The role of efflux machinery in carbapenem resistance

The sensitivity of the tested isolates to imipenem was established via microtitre plate broth dilution method with and without the addition of efflux pump inhibitors; carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma-aldrich, St. Louis, USA) at a concentration of 40 µg/mL and Phe-Arg-b-naphthylamide (PAbN; PAbN; Sigma-aldrich, St.

Louis, USA) at a concentration of 50 µg/mL [17-18]. The MIC of the imipenem/inhibitor was compared to the MIC of imipenem alone using paired T test with $p < 0.01$ was considered significant.

Real-time PCR of porin (OprD) and efflux pump

P. aeruginosa isolates resistant to carbapenems were grown to the mid-log phase (OD_{600}: 0.4-0.5). Cell pellets were collected and total RNA was extracted using Triazole reagent (Sigma, USA). RNA was purified and cDNA was synthesized using Quanti Tect Rev. Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Relative expression of efflux pump gene *mexB* and the outer membrane protein gene *oprD* were determined by Real-time PCR (RT-PCR) utilizing FIREPol EvaGreen, qPCR Mix (Solis BioDyne, Tartu, Estonia), with RotorGene 6000 Real-Time PCR system (Corbett Robotics Pty Ltd, Brisbane, Australia).

The expression level of both *mexB* and *oprD* was relatively normalized to the expression of *rpoD* (housekeeping gene). Overexpression of efflux pump MexAB-OprM was established when the expression of *mexB* was 2-3 folds its expression level in the wild isolate PAO1 [19-20]. The expression level of *oprD* was also calibrated relative to PAO1 [21].

Molecular characterization of P. aeruginosa

PFGE was performed as illustrated previously by Lopes *et al*., 2015 [22]. In brief, genomic DNA was digested with 50 U of *SpeI* (New England Biolabs, Massachusetts, USA). DNA fragments were separated by PFGE using a CHEF-DR III system. The running conditions include initial switch time of 2.2 seconds, running time for 22 hours, gradient of 6 volt/cm, angle of 120, temperature of 14 ºC and a final switch time of 54.2 seconds. Gels were stained with 1 µg/mL of ethidium bromide (Sigma Life Science) for 30 minutes and gel images were captured using UV gel image acquisition camera, Gel Doc XR (Bio-Rad Laboratories, Carlsbad, USA). PFGE pattern comparison is based on published procedures [23]. DNA relatedness was detected based on DICE coefficient with 4% optimization and 2% tolerance using BioNumerics Software (Applied Maths, Sint-Martens-Latem, Belgium).

Statistical analysis

The mean and standard deviation of the mean (SD; three replicates) were calculated using Microsoft Excel. All results were presented as mean ± SD. Statistical tests were applied as described under each experiment.

Table 1. Sequences of the primers used to detect carbapenem resistance mechanism in *P. aeruginosa* clinical isolates.

Gene type	Gene name	Type of primer	Nucleotide sequence	Melting Temp	Amplicon size (bp)
Reference gene	rpoD	F	5`–CGAACTGCTTGCCGACTT–3`	56°C	131
		R	5`–GCGAGAGCCTCAAGGATAC–3`		
β-lactamases / carbapenemases genes	NDM-1	F	5`– ACTTCCTATCTCGACATGC –3`	52°C	133
		R	5`– TGATCCAGTTGAGGATCTG –3`		
	NDM-1F	F	5` – TGTTATGGAGCAGCAACGATG – 3`	62°C	795
		R	5` – AAAGTCCCGCTCCAACGATT – 3`		
	KPC	F	5`– ATTCGCTAAACTCGAACAG –3`	50°C	130
		R	5`– AAGAAAGCCCTTGAATGAG –3`		
	VIM-1	F	5`– TGTTATGGAGCAGCAACGATG –3`	55°C	920
		R	5`– AAAGTCCCGCTCCAACGATT –3`		
	VIM-2	F	5`– GTCTATTTGACCGCGTCTATC–3`	55°C	774
		R	5`– CTACTCAACGACTGAGCGAT–3`		
	IMP	F	5`– GTTAGTCACTTGGTTTGTG –3`	50°C	103
		R	5`– CGAGAATTAAGCCACTCTA –3`		
	OX	F	5`– AAGTGTGCAACGCAAATGGC –3`	55°C	137
		R	5`– CTGTTCCAGATCTCCATTCC –3`		
Efflux pump and outer membrane genes	MexB	F	5`–GAAGAACTTCCTCATGGTGGTC–3`	58°C	101
		R	5`–AGAGTGGGTCCTGGATGTT–3`		
	OprD	F	5`– CAAGAGCGCCGATTTCATTG –3`	58°C	92
		R	5`–GGCGATAGATGTCTTCGAGTTC–3`		

F: forward, R: reverse, Temp: temperature, bp: base pair.

Results

Antimicrobial susceptibility test

Ninety *P. aeruginosa* isolates were purified and identified biochemically. The isolates were obtained from different clinical sources including urine, pus swabs and sputum. *P. aeruginosa* isolates were collected from University Hospital (26 isolates), Chest Hospital (13 isolates), Urology and Nephrology Center (24 isolates), Burns and Cosmetics Center (11 isolates), and International Hospital (16 isolates). The antimicrobial susceptibility testing revealed that sixteen isolates (17.7%) were resistant to imipenem. All imipenem resistant isolates were also meropenem resistant except isolate number 53 (Table 2). Most imipenem resistant isolates were resistant to other antimicrobials including gentamicin (56.25%), levofloxacin (81.25%), ceftazidime (62.5%) and amikacin (62.5%). Eight isolates (50%) were defined to be multiple drug resistant (Table 2). The MICs of imipenem ranged from 32 to 2048 µg per mL and the MICs values of meropenem resistant isolates ranged from 16 to 256 µg per mL (Table 3).

MHT

Detection of carbapenemases by Modified Hodge test revealed that twelve isolates were carbapenemase producers (Table 3).

PCR of carbapenemases encoding genes

PCR identified the presence of bla_{VIM-1} in 12 isolates, bla_{VIM-2} carbapenemases in 12 isolates, whereas eight isolates harbored both genes. Eight *P. aeruginosa* isolates possessed bla_{NDM-1} (Table 3). bla_{NDM-1} was detected with bla_{VIM-1} in isolates 53 and 79, with bla_{VIM-2} in isolates 29 and 75, and with both genes in isolates 11, 13, 37, and 41. On the other hand, bla_{OXA}, bla_{IMP} and bla_{KPC} were not detected in the carbapenem resistant isolates.

Effects of efflux pump inhibitors on carbapenem resistance

When efflux pump inhibitors CCCP (40 µg per mL) and PAbN (50 µg per mL) were added to imipenem, a significant reduction in MICs of imipenem was detected (Table 3). Treating the bacteria with efflux pump inhibitors alone without imipenem showed no effect on bacterial growth. In addition, the efflux inhibitory effect of PAbN was more significant compared to CCCP.

Expression of efflux pump and outer membrane protein

RT-PCR analysis of *mexB* revealed that seven carbapenem-resistant isolates exhibited high expression level of *mexB* (Table 3). Loss of outer membrane OprD was exhibited by seven isolates including sputum isolates number 13, 28 and 29, urine isolates number 41, 53, and 56 and isolate number 89 purified from wound (Table 3).

Table 2. Source of the carbapenem resistant isolates and their antimicrobial sensitivity patterns.

Isolate code	Hospital	Source	Sensitivity pattern					
			Ceftazidime	Imipenem	Meropenem	Gentamicin	Amikacin	Levofloxacin
11	UH	Sputum	S	R	R	S	S	R
13	UH	Sputum	R	R	R	R	R	R
28	UH	Sputum	R	R	R	I	S	R
29	CH	Sputum	R	R	R	R	R	S
36	UH	Urine	R	R	R	R	R	R
37	UNC	Urine	I	R	R	R	R	R
41	UNC	Urine	R	R	R	R	R	R
53	UNC	Urine	I	R	S	S	S	S
56	UNC	Urine	I	R	R	S	R	S
75	BCC	Pus	R	R	R	R	R	R
78	BCC	Pus	R	R	R	S	I	R
79	BCC	Pus	R	R	R	S	S	R
83	UH	Pus	S	R	R	S	S	R
85	UH	Pus	R	R	R	R	R	R
86	IH	Pus	S	R	R	R	R	R
89	BCC	Pus	R	R	R	R	R	R

UH: University Hospital, CH: Chest Hospital, UNC: Urology and Nephrology Center, BCC: Burns and Cosmetics Center, IH: International Hospital, R: resistant, S: sensitive, I: intermediate.

Figure 1. Dendrogram of PFGE-*Spe I* profiles of the 16 *P. aeruginosa* clinical isolates.

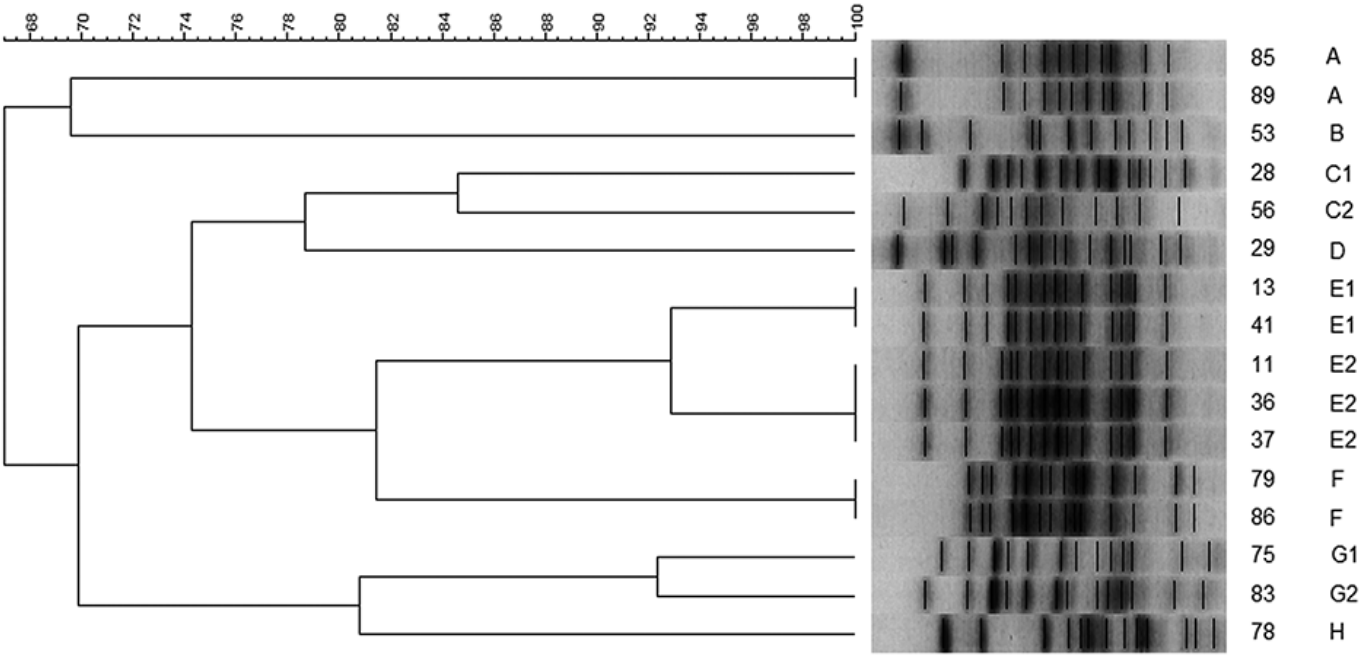

Table 3. Characterization of *P. aeruginosa* clinical isolates.

Isolate code	MHT	PCR analysis *bla*NDM-1, *bla*VIM-1, *bla*VIM-2, *bla*KPC, *bla*OXA, *bla*IMP	MIC MEM µg mL^{-1}	MIC IPM µg mL^{-1}	MIC IPM /PAbN µg mL^{-1}	MIC IPM / CCCP µg mL^{-1}	Expression by RT-PCR	
							MexB	*OprD*
11	-	*bla*NDM-1, *bla*VIM-1, *bla*VIM-2,	16	128	2	2 ± 0	ND	35.260 ± 0.01
13	+	*bla*NDM-1, *bla*VIM-1, *bla*VIM-2,	16	64	< 1	32	0.191 ± 0.02	0.076 ± 0.002
28	-	*bla*VIM-1	16	32	8	16	ND	0.030 ± 0.015
29	+	*bla*NDM-1, *bla*VIM-2	32	256	< 1	128	0.0421 ± 0.01	0.675 ± 0.021
36	+	*bla*VIM-1, *bla*VIM-2	128	2048	4	512	3.031 ± 0.03	43.111 ± 0.67
37	+	*bla*NDM-1, *bla*VIM-1, *bla*VIM-2	128	512	< 1	512	2.713 ± 0.03	1.2145 ± 0.01
41	+	*bla*NDM-1, *bla*VIM-1, *bla*VIM-2	64	2048	256	512	13.737 ± 0.02	0.005 ± 0.001
53	-	*bla*NDM-1, *bla*VIM-1	2	64	4	16	ND	0.167 ± 0.003
56	+	*bla*VIM-1	256	512	8	512	54.192 ± 0.07	0.020 ± 0.01
75	+	*bla*NDM-1, *bla*VIM-2	64	512	32	256	0.37 ± 0.01	11.158 ± 0.21
78	+	*bla*VIM-1, *bla*VIM-2	128	256	128	256	94.353 ± 0.1	17.148 ± 0.41
79	+	*bla*NDM-1, *bla*VIM-1	128	256	512	256	0.758 ± 0.015	26.909 ± 0.70
83	+	*bla*VIM-1, *bla*VIM-2	64	256	128	256	335.461 ± 0.6	14.123 ± 0.86
85	+	*bla*VIM-2	64	128	32	128	1.248 ± 0.01	62.250 ± 0.99
86	+	*bla*VIM-1, *bla*VIM-2	32	128	32	64	0.674 ± 0.02	28.640 ± 0.53
89	-	*bla*VIM-2	128	256	128	256	4.659 ± 0.05	0.002 ± 0.001

MHT; Modified Hodge test, PCR; polymerase chain reaction, MIC: minimum inhibitory concentration, MEM: meropenem, IPM: imipenem, PAbN: Phe-Arg-b-naphthylamide, CCCP: carbonyl cyanide m- chlorophenyl hydrazone, RT-PCR: Real-time polymerase chain reaction, ND: not detected

Molecular characterization of P. aeruginosa

PFGE of *SpeI*-digested genomic DNA from 16 *P. aeruginosa* isolates resulted in 11 distinct pulsed-field profiles (PFPs) comprising 11–15 restriction fragments with 70% similarity. The 11 PFPs were identified as A, B, C1, C2, D, E1, E2, F, G1, G2 and H with two *P. aeruginosa* isolates belonging to related clusters C1 and C2, five isolates to E1 and E2 and two isolates to G1 and G2 (Figure 1). Three *P. aeruginosa* isolates belonged to clusters B, D and H with one isolate in each cluster. Interestingly, we found that the isolates with multiple carbapenemases genes (bla_{NDM-1}, bla_{VIM-1}, bla_{VIM-2}), correspond to the profiles E1 and E2 (Table 4).

Discussion

Antimicrobial resistance is a significant driver of high mortality and morbidity associated with bacterial infections all over the world. Carbapenems have been used as the last choice for treatment of many bacterial infections [24]. The current study report carbapenem resistance in 17% of the tested isolates. The highest number of carbapenem-resistant isolates has been identified among the isolates from pus swab (7/17) (Table 2). Moreover, eight carbapenem resistant isolates (50%) are multi-drug resistant to three classes of antimicrobial agents (Table 2). Overall, high resistance to extended-spectrum β-lactams and carbapenems were reported in *P. aeruginosa* worldwide [4]. A rapid dissemination of carbapenemases enzymes has been reported in different countries, including India, Pakistan, Australia, Germany, Belgium, Canada, Kenya, Sultanate of Oman, and United States [25-26].

Our findings reveal that all carbapenem-resistant *P. aeruginosa* isolates harbor carbapenemase genes especially MBLs (bla_{NDM-1}, bla_{VIM-1} and bla_{VIM-2}) (Table 3). Herein, bla_{VIM-1} (75%) and bla_{VIM-2} (75%) are the most commonly detected carbapenemase genes (Table 3). This result coincides with the high prevalence of bla_{VIM-2} among *P. aeruginosa* isolates in Egypt [27-28]. Furthermore, a serious outbreak of bla_{VIM} has been reported in France, Greece, Costa Rica, and Saudi Arabia [29-32]. In addition to VIM, NDM is another leading cause of a high-level of resistance to all carbapenems. *P. aeruginosa* isolates with bla_{NDM-1} have been detected also in Serbia [33], France [34], and India [35]. NDM-1 was reported in Egypt with low prevalence among *P. aeruginosa* isolates where only two out of 33 carbapenem-resistant *P. aeruginosa* isolates harbor NDM [36]. Here, we report an increase to 50% of carbapenem-resistant *P. aeruginosa* isolates that harbor NDM. Also, we detected *P. aeruginosa* isolates with more than one carbapenemase gene, of which four of them possessed three genes; bla_{NDM-1}, bla_{VIM-1} and bla_{VIM-2}. Few studies have reported the coexistence of carbapenemases encoding genes in the same *P. aeruginosa* isolate such as KPC and IMP-8 in Puerto Rico [37], KPC and VIM in Colombia [38] and SPM-1, KPC-2 and VIM-2 in Brazil [39].

The MICs of imipenem in this study were very high, reaching 2048 µg per mL (Table 3). Therefore, these studied *P. aeruginosa* isolates could have other resistance mechanisms. Overexpression of efflux proteins and decreased drug permeability may also affect the activity of carbapenems on *P. aeruginosa* [1,5]. A significant decrease in MIC of imipenem in combination with 50 µg per mL PAbN or 40 µg per mL CCCP compared to that of imipenem alone was determined. These results point out the contribution of the active efflux pump to the emergence of carbapenem-resistant *P. aeruginosa*. Moreover, real-time PCR analysis confirmed overexpression of genes

Table 4. Clonal relatedness of carbapenemases producing *P. aeruginosa* obtained by PFGE.

PFGE clone	Number of isolates	Carbapenemase gene
C1	1	*(n = 2)* bla_{VIM-1}
C2	1	
A	2	*(n = 2)* bla_{VIM-2}
E2	1	*(n = 4)* bla_{VIM-1}, bla_{VIM-2}
H	1	
G2	1	
F	1	
B	1	bla_{NDM-1}, bla_{VIM-1} *(n = 2)*
F	1	
D	1	bla_{NDM-1}, bla_{VIM-2} (*n = 2*)
G1	1	
E1	2	bla_{NDM-1}, bla_{VIM-1}, bla_{VIM-2} *(n = 4)*
E2	2	

PFGE: Pulsed field gel electrophoresis.

involved in active efflux machinery in seven isolates (43.7%) with 3-300 folds overexpression of *mexB* relative to PAO1. Upregulation of *mexB* was observed in *P. aeruginosa* isolates with elevated MICs of imipenem (128- 2048 µg/mL) and meropenem (64- 256 µg/mL). These findings indicate the role of active efflux pump in carbapenem resistance. In previous research, overexpression of MexAB-OprM has been identified as the primary mechanism of meropenem resistance [40].

Moreover, both meropenem and imipenem utilize the OprD pathway for entry into *Pseudomonas* cell [5]. Therefore, reduced OprD expression also contributes to reduced susceptibility to carbapenems in *P. aeruginosa* [41]. In the current study, seven isolates (43.7%) had low OprD expression, five of them were highly resistant to imipenem (MIC 128- 2048 µg/mL) and two were resistant to meropenem (MIC 64- 256 µg/mL). In the study of Yi *et al*., 2006 [40], imipenem resistance is mainly mediated by OprD deficiency. In agreement with that, Kim and coauthors have recently reported that OprD inactivation is the most common mechanism of carbapenem resistance in *P. aeruginosa* isolated from Korean patients [42].

In this study, combined mechanisms of carbapenem resistance were recorded in most *P. aeruginosa* isolates. It was found that 56% of the resistant isolates exhibited two mechanisms of resistance to carbapenem and 25% of the resistant isolates possessed three mechanisms. Recently, the same observation of several resistance mechanisms to carbapenem was reported in *P. aeruginosa* isolates from Taiwan [43] and Spain [44].

Another significant observation in our study is the close genetic relation of isolates from different hospitals as indicated by PFGE (Figure 1). Isolates 85 and 89 belong to clone A and they were collected from Mansoura University Hospital and Burns and Cosmetics Center, respectively. Similarly, isolates 79 and 86 of clone F were isolated from Burns and Cosmetics and Mansoura International Hospital, respectively. Moreover, isolates 36 and 37 belong to clone E2 and they were isolated from University Hospital and Urology and Nephrology Center, respectively. These results suggest a possibility of microbial dissemination from one hospital to another rather than intrahospital transmission of these isolates.

Conclusion

In the present study, carbapenemases enzymes were identified in carbapenem resistant isolates with a high prevalence of $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$ and $bla_{\text{NDM-1}}$. Most isolates harbored two or three carbapenemase genes. Induction of the efflux machinery and reduced expression of OprD are also involved in carbapenem resistance of *P. aeruginosa*. Moreover, the emergence of similar clones of carbapenem-resistant *P. aeruginosa* suggests microbial dissemination of the isolates with multiple carabapenemases. Hence, active surveillance of carbapenemase enzymes in addition to other carbapenem resistance mechanisms and enforcement of appropriate infection control precautions are obligatory to inhibit the dissemination of carbapenem-resistant *P. aeruginosa* infection.

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References

1. Lister PD, Wolter DJ, Hanson ND (2009) Antibacterial-resistant *Pseudomonas aeruginosa*: Clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev 22: 582-610.
2. Hirsch EB, Tam, VH (2010) Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. Expert Rev Pharmacoecon Outcomes Res 10: 441-451.
3. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA (2011) Carbapenems: Past, Present, and Future. Antimicrob Agents Chemother 55: 4943-4960.
4. Hong DJ, Bae IK, Jang IH, Jeong SH, Kang HK, Lee K (2015) Epidemiology and characteristics of metallo-β-lactamase-producing *Pseudomonas aeruginosa*. Infect Chemother 47: 81-97.
5. Livermore DM (2001) Of *Pseudomonas*, porins, pumps and carbapenems. J Antimicrob Chemother 47: 247-250.
6. Villegas MV, Lolans K, Correa A, Kattan JN, Lopez JA, Quinn JP (2007) First identification of *Pseudomonas aeruginosa* isolates producing a KPC-type carbapenem-hydrolyzing beta-lactamase. Antimicrob Agents Chemother 51: 1553-1555.
7. Pollini S, Maradei S, Pecile P, Olivo G, Luzzaro F, Docquier JD, Rossolini GM (2013) FIM-1, a new acquired metallo-β-lactamase from a *Pseudomonas aeruginosa* clinical isolate from Italy. Antimicrob Agents Chemother 57: 410-416.
8. Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, Alberti S, Bush K, Tenover FC (2001) Novel carbapenem-hydrolyzing β-Lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae.* Antimicrob Agents and Chemother 45: 1151-1161.
9. Nordmann P, Cuzon G, Naas T (2009) The real threat of KPC carbapenemase-producing bacteria. Lancet Infect Dis 9: 228-236.
10. Li XZ, Nikaido H (2009) Efflux-mediated drug resistance in bacteria: an update. Drugs 69: 1555-1623.
11. Yang D, Guo Y, Zhang Z (2009) Combined porin loss and extended spectrum β-lactamase production is associated with an increasing imipenem minimal inhibitory concentration in clinical *Klebsiella pneumoniae* strains. Curr Microbiol 58: 366-370.

12. Ocampo-Sosa AA, Cabot G, Rodríguez C, Roman E, Tubau F, Macia MD, Moya B, Zamorano L, Suárez C, Peña C, Domínguez MA, Moncalián G, Oliver A, Martínez-Martínez L (2012) Alterations of OprD in carbapenem-intermediate and -susceptible strains of *Pseudomonas aeruginosa* isolated from patients with bacteremia in a Spanish multicenter study. Antimicrob Agents and Chemother 56: 1703-1713.
13. Quale J, Bratu S, Gupta J, Landman D (2006) Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. Antimicrob Agents Chemother 50: 1633-1641.
14. Cheesbrough M (2006). District Laboratory Practice in Tropical Countries Part II, 2nd edition. Cambridge: Cambridge University Press195 p.
15. Clinical and Laboratory Standards Institute (CLSI) (2015) Performance standards for antimicrobial susceptibility testing, 25th informational supplement. CLSI document M100-S25 (ISBN 1-56238-990-4).
16. Lee K, Chong Y, Shin HB, Kim YA, Yong D, Yum JH (2001) Modified Hodge and EDTA-disk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. Clin Microbiol Infect 7: 88-91.
17. Talebi-Taher M, Majidpour A, Gholami A, Rasouli-kouhi S, Adabi M (2016) Role of efflux pump inhibitor in decreasing antibiotic cross-resistance of *Pseudomonas aeruginosa* in a burn hospital in Iran. J Infect Dev Ctries 10: 600-604. doi: 10.3855/jidc.7619.
18. El-Shaer S, Shaaban M, Barwa R, Hassan R (2016) Control of quorum sensing and virulence factors of *Pseudomonas aeruginosa* using phenylalanine arginyl b-naphthylamide. J Med Microbiol 65: 1194–1204
19. Li XZ, Nikaido H, Poole K (1995) Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 39: 1948-1953.
20. Mesaros N, Glupczynski Y, Avrain L, Caceres NE, Tulkens PM, Van Bambeke F (2007) A combined phenotypic and genotypic method for the detection of Mex efflux pumps in *Pseudomonas aeruginosa*. J Antimicrob Chemother 59: 378-386.
21. Pirnay JP, De Vos D, Mossialos D, Vanderkelen A, Cornelis P, Zizi M (2002) Analysis of the *Pseudomonas aeruginosa oprD* gene from clinical and environmental isolates. Environ Microbiol 4: 872-882.
22. Lopes BS, Al-Agamy MH, Ismail MA, Shibl AM, Al-Qahtani AA, Al-Ahdal M N, Forbes KJ (2015) The transferability of blaOXA-23 gene in multidrug-resistant Acinetobacter baumannii isolates from Saudi Arabia and Egypt. Int J Med Microbiol 305: 581-588.
23. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33: 2233-2239.
24. El-Mahdy TS (2014) Identification of a novel metallo-β-lactamase VIM-28 located within unusual arrangement of class 1 integron structure in *P. aeruginosa* isolates from Egypt. Jpn J Infect Dis 67: 382-384.
25. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt FA, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U., Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S., Warner M, Welfare W, Livermore DM, Woodford N (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis 10: 597-602.
26. Poirel L, Fortineau N, Nordmann P (2011) International transfer of NDM-1-producing *Klebsiella pneumoniae* from Iraq to France. Antimicrob Agents Chemother 55: 1821-1822.
27. Diab M, Fam N, El-Said M, El-Dabaa E, El-Defrawy I, Saber M (2013) Occurrence of VIM-2 Metallo-β- lactamases in imipenem resistant and susceptible *Pseudomonas aeruginosa* clinical isolates from Egypt. Afr J Microbiol Res 7: 4465-4472.
28. Zafer MM, Al-Agamy MH, El-Mahallawy HA, Amin MA, Ashour SE (2015) Dissemination of VIM-2 producing *Pseudomonas aeruginosa* ST233 at tertiary care hospitals in Egypt. BMC Infect Dis 15: 122.
29. Cuzon G, Bonnin RA, Nordmann P (2013) First identification of novel NDM carbapenemase, NDM-7, in *Escherichia coli* in France. PLoS One. 8: e61322.
30. Tsakris A, Pournaras S, Woodford N, Palepou M-F, Babini GS, Douboyas J, Livermore DM (2000) Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. J Clin Microbiol 38: 1290-1292.
31. Toval F, Guzmán-Marte A, Madriz V, Somogyi T, Rodríguez C, García F (2015) Predominance of carbapenem-resistant *Pseudomonas aeruginosa* isolates carrying *bla*IMP and *bla*VIM metallo-β-lactamases in a major hospital in Costa Rica. J Med Microbiol 64: 37-43.
32. Abdalhamid B, Elhadi N, Alabdulqader N, Alsamman K, Aljindan R (2016). Rates of gastrointestinal tract colonization of carbapenem-resistant Enterobacteriaceae and *Pseudomonas aeruginosa* in hospitals in Saudi Arabia. New Microbes New Infect 29: 77-83.
33. Jovcic B, Lepsanovic Z, Suljagic V, Rackov G, Begovic J, Topisirovic L, Kojic M (2011) Emergence of NDM-1 metallo-beta-lactamase in *Pseudomonas aeruginosa* clinical isolates from Serbia. Antimicrob Agents Chemother 55: 3929-3931.
34. Janvier F, Jeannot K., Tessé S, Robert-Nicoud M, Delacour H, Rapp C, Mérens A (2013) Molecular characterization of *bla*NDM-1 in a sequence type 235 *Pseudomonas aeruginosa* isolate from France. Antimicrob Agents Chemother 57: 3408-3411.
35. Khajuria A, Praharaj AK, Kumar M, Grover N (2013) Emergence of NDM- 1 in the clinical isolates of *Pseudomonas aeruginosa* in India. J Clin Diagn Res 7: 1328-1331.
36. Zafer MM, Amin M, El Mahallawy H, Ashour MS, Al Agamy M (2014) First report of NDM-1-producing *Pseudomonas aeruginosa* in Egypt. Int J Infec Dis 29: 80-81.
37. Correa A, Montealegre MC, Mojica MF, Maya JJ, Rojas LJ, De La Cadena EP, Ruiz SJ, Recalde M, Rosso F, Quinn JP, Villegas MV (2012) First report of a *Pseudomonas aeruginosa* isolate co-harboring KPC and VIM carbapenemases. Antimicrob Agents Chemother 56: 5422-5423.
38. Martnez T, Vzquez GJ, Aquino EE, Ramrez-Ronda R, Robledo IE (2012) First report of a *Pseudomonas aeruginosa* clinical isolate co-harboring KPC-2 and IMP-18 carbapenemases. Int J Antimicrob Agents 39: 542-543.
39. Rizek C, Fu L, Dos Santos LC, Leite G, Ramos J, Rossi F, Guimaraes T, Levin AS, Costa SF (2014) Characterization of carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates, carrying multiple genes coding for this antibiotic resistance. Ann. Clin Microbiol Antimicrob 13: 43.
40. Yi MY, Wang PY, Huang HJ, Liu YC (2006) The roles of active efflux system overexpression and outer membrane

protein OprD deficiency or loss in carbapenem resistance of *Pseudomonas aeruginosa*. Zhonghua yi xue za zhi. 86: 457-462. [Article in Chinese].
41. Gutierrez O, Juan C, Cercenado E, Navarro F, Bouza E, Coll P, Perez JL, Oliver A (2007) Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. Antimicrob Agents Chemother 51: 4329-4335.
42. Kim CH, Kang HY, Kim BR, Jeon H, Lee YC, Lee SH, Lee JCC (2016) Mutational inactivation of OprD in carbapenem-resistant *Pseudomonas aeruginosa* isolates from Korean hospitals. J Microbiol 54: 44-49.
43. Kao CY, Chen SS, Hung KH, Wu HM, Hsueh PR, Yan JJ, Wu JJ (2016) Overproduction of active efflux pump and variations of OprD dominate in imipenem resistant *Pseudomonas aeruginosa* isolated from patients with bloodstream infections in Taiwan. BMC Microbiology 16: 107.
44. Solé M, Fàbrega A, Cobos-Trigueros N, Zamorano L, Ferrer-Navarro M, Ballesté-Delpierre C, Reustle A, Castro P, Nicolás JM, Oliver A, Martínez J, Vila J (2015) In vivo evolution of resistance of *Pseudomonas aeruginosa* strains isolated from patients admitted to an intensive care unit: mechanisms of resistance and antimicrobial exposure. J Antimicrob Chemother 70: 3004-3013.

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