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ORIGINAL ARTICLE

MexAB-OprM and MexXY-OprM efflux pumps overexpression; additional mechanism for carbapenems resistance among nosocomial *Pseudomonas aeruginosa* isolates

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ABSTRACT

Background: *Multidrug-resistant pathogens have been on the rise during the last few years. Pseudomonas aeruginosa is commonly encountered in nosocomial infections with remarkable ability to develop antimicrobial resistance of which carbapenems are of great concern.* ***Objectives:*** *To explore the role of MexAB-OprM and MexXY-OprM efflux pumps overexpression as carbapenems resistance mechanisms among nosocomial P. aeruginosa isolates at both Menoufia and Kasr Al Ainy University Hospitals by phenotypic and molecular characterization methods.* ***Methodology:*** *A total of 120 P. aeruginosa isolates were collected from patients with hospital-acquired infections and subjected to antibiotic susceptibility testing by the Kirby-Bauer disk diffusion method. Carbapenems-resistant isolates were selected and investigated phenotypically for the contribution of MexAB-OprM and MexXY-OprM efflux pumps by both disk synergy and MIC reduction assays with cyanide-m-chlorophenyl hydrazone (CCCP) as an efflux pump inhibitor. Real time PCR assay verified the existence of mexA and mexX genes as regulators of MexAB-OprM and MexXY-OprM overexpression. Laboratory results were correlated with data regarding patients' clinical findings as well as risk factors.* ***Results:*** *Out of 120 P. aeruginosa isolates, 88 (73.3%) isolates were carbapenems-resistant of which 100% were MDR isolates. The highest resistance rate was for piperacillin and piperacillin/tazobactam (100% for each) and the lowest rate was seen against colistin (7.5%).The RT-PCR assay revealed that, 54/88 (61.3%) P. aeruginosa isolates harbored the target genes: 21 isolates (38.9%) were positive for mexA alone, 12 isolates (22.2%) were positive for mexX alone and 21 isolates (38.9%) showed co-existence of the two genes. In relation to PCR results, the sensitivity, specificity and accuracy of CCCP disk synergy test respectively were 46%, 94% and 64.8% while, those for MIC method were 88.9%, 55.9% and 76.1% respectively.* ***Conclusion****: Carbapenems resistance mediated by the overexpression of efflux pumps has also now emerged. Early recognition of this resistance mechanism to allow the use of alternative b-lactams is imperative.*

INTRODUCTION

Emergence of multidrug resistance among *P. aeruginosa* clinical isolates is a serious challenge for the medical community. Although previously considered as an opportunistic pathogen, MDR *P.aeruginosa* is now involved in a wide range of difficult to treat - nosocomial infections [1,2].

Carbapenems resistance in *P. aeruginosa* has become an important threat all over the world[2]. For *P. aeruginosa*, carbapenems resistance is multifactorial and is mediated by both intrinsic and acquired mechanisms. Intrinsic refers to resistance that is a consequence of genetically-encoded mechanisms and acquired refers to acquisition of additional mechanisms or mutational changes[3]. Plasmid and/or integron-mediated carbapenemases, over expression of efflux systems and reduction of outer membrane porins have all been addressed as contributory factors [4].

Efflux pump systems are extremely important mechanisms for emergence of MDR *P. aeruginosa* belonging to resistance-nodulation-cell division (RND) family[5]. The RND multidrug efflux systems operate as tripartite systems consisting of a cytoplasmic membrane-associated RND transporter (e.g. MexB, MexD, MexF and MexY), periplasmic membrane fusion protein (MFP) e.g. MexA, MexE and MexX and an outer membrane protein (e.g. OprM, OprJ, and OprN). Substrates for these efflux pumps are commonly quinolones, antipseudomonal penicillins, cephalosporins, aminoglycosides and carbapenems[6].

The genome of *P. aeruginosa* possesses at least 12 structural genes for the RND efflux systems, of which four are clinically-important (i.e. MexABOprM,

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MexCD-OprJ, MexEF-OprN and MexXY-OprM) [5]. The pumps, especially the RND family, have received particular recent attention because they can extrude multiple structurally unrelated components, and thus are strongly involved in multidrug resistance [7].

Diagnosis of efflux-mediated resistance provides additional help for both routine clinical analysis and epidemiological studies [8]. Efflux pump inhibitors (EPIs) have been under investigation as an alternative to the development of new antibiotics for treatment of *P. aeruginosa* infection [9].

The specific detection of efflux pumps has long relied on western blot or northern blot analysis, but these methods cannot easily be implemented in the clinical laboratory [10]. Nucleic acid-based diagnostics are gradually replacing culture based biochemical and immunological assays because of their high level of specificity. Semi-quantitative reverse transcription PCR (RT-PCR) or quantitative real-time PCR, have been successfully used for detecting the expression of efflux pumps in *P. aeruginosa* [11].

The current study aimed to investigate the role of MexAB-OprM together with MexXY-OprM efflux pumps overexpression in the development of carbapenems resistance among nosocomial *P. aeruginosa* isolates from both MUHs and Kasr Al Ainy Hospitals by phenotypic and molecular characterization methods.

METHODOLOGY

Collection, identification and storage of *P. aeruginosa* isolates:

The study involved a total of 120 *P. aeruginosa* isolates recovered from various clinical samples collected from patients admitted to different departments and ICUs of the two participating hospitals during the period from June 2019 to January 2020. *P. aeruginosa* isolates were identified by the standard microbiological methods [12]and were subjected to the following:

Antibiotic susceptibility testing:

By the Kirby-Bauer disk diffusion method on Mueller Hinton (MH) agar (High Media, India) for all *P. aeruginosa* isolates against different antimicrobial agents (Oxoid, UK) (table-2). Zone diameters were interpreted as per CLSI/2019 guidelines [13]. Multi-drug resistance (MDR) was defined as non-susceptibility to at least one agent in three or more antimicrobial categories as per the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the US Food and Drug Administration [14, 15].

Bacterial isolates selection:

Considered as one of the most common carbapenems resistance mechanisms among *P. aeruginosa* isolates[5], class A and class B carbapenemase- producing activity was excluded respectively by applying both meropenem-boronic acid and meropenem- EDTA synergy tests [16,17] and only carbapenems- resistant carbapenemase- negative isolates were involved in the current study (88 *P. aeruginosa* isolates). Additionally, *P. aeruginosa* ATCC 27853 and PAO1 were used as reference strains to determine the baseline level of MexAB-OprM and MexXY-OprM efflux pumps expression.

Phenotypic characterization of MexAB-OprM and MexXY-OprM efflux pumps:

Synergy experiments were applied using meropenem and the efflux pump inhibitor carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) (Sigma-Aldrich, St. Louis, USA) which is used to detect efflux pump overexpression in the isolated *P. aeruginosa* strains[18]. CCCP was incorporated in MH agar (High Media, India) and meropenem susceptibility testing by disk diffusion and agar dilution was performed in parallel in agar plates with and without CCCP as follows:

A- CCCP synergy disk test:

Briefly, MH agar plates containing CCCP at a concentration of 12.5 µM were prepared. 0.5 McFarland bacterial suspension of the test isolate was prepared and inoculated with a sterile cotton swab on a CCCP-supplemented plate and in parallel on a CCCP-free plate. Meropenem disk (10µg, Oxoid, UK) was placed on both plates for each inoculation and the plates were incubated at 37^{o}C for 18-24h. The test was considered positive when synergy between meropenem and CCCP was observed on the CCCP-supplemented plates[18] (fig.1).

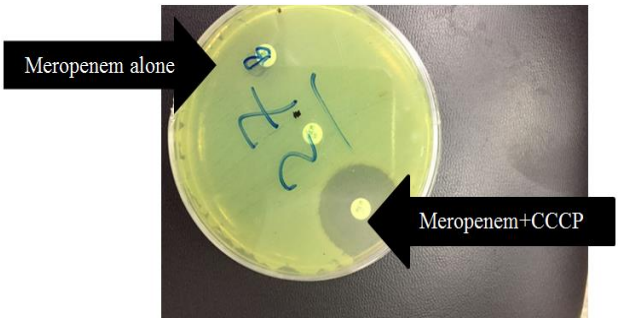

Fig. 1: CCCP disk synergy test

B- Minimum inhibitory concentration (MIC) method:

Firstly, MICs for meropenem were determined by the agar dilution method and then MIC analyses of meropenem were performed again in the presence of CCCP for all resistant strains. CCCP was incorporated in MH agar at concentrations of 12.5 µM, and MIC reduction testing was performed by the twofold serial dilution method using a final inoculum of 10^6 cells/mL in agar plates with CCCP [19]. *P. aeruginosa* ATCC 27853 and PAO1 were used as reference strains. Results were interpreted as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI; 2019)[13] (fig.2).

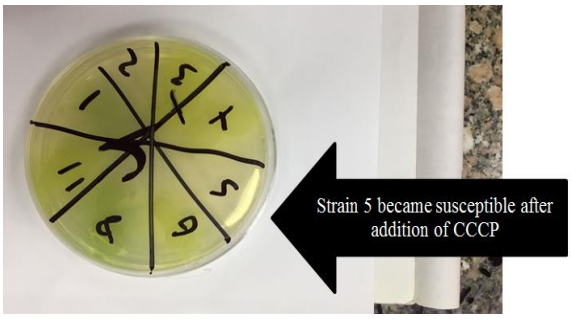

Fig. 2: MIC reduction assay

Genotypic characterization of efflux pumps overexpression by Real-time PCR:

The selected 88 carbapenems-resistant *P. aeruginosa* isolates were investigated by RT–PCR for the overexpression of *mexA* and *mexX* genes which are representative of MexAB-OprM and MexXY-OprM efflux pumps respectively. Overexpression level was determined in relation to the quality control strain (PAO1/*rspL* gene). Primers used in the study are shown in the following table:

Gene	Primers (5'- 3')	Reference
mexA	**Forward:** AACCCGAACAACGAGCTG	**20**
	Reverse: ATGGCCTTCTGCTTGACG	
Probe	[DFAM]CATGTTCGTTCACGCGCAGTTG[DTAM]	
mexX	**Forward:** GGCTTGGTGGAAGACGTG	
	Reverse: GGCTGATGATCCAGTCGC	
Probe	[DFAM]CCGACACCCTGCAGGGCC[DTAM]	

RNA extraction and cDNA synthesis:

Total RNA was extracted from the cultured medium using QIAGEN RNeasy Mini Kit (Qiagen, Tokyo, Japan), and residual DNA was removed by adding DNase I using QIAGEN RNase-Free DNase Set (Qiagen) according to the manufactures instructions. For cDNA synthesis, each 20µl reaction contained 1 µg of total RNA, 10 µg of random hexamer, 1× first strand buffer (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2; Invitrogen, Tokyo, Japan), 0.5 mM dNTP, 10U of RNase inhibitor (Invitrogen), and 200U of Super Script II (Invitrogen). cDNA synthesis was performed in a PCR Thermal Cycler (Takara, Kyoto, Japan). ***Quantitative real-time PCR assay***: The LightCycler (Roche, Tokyo, Japan) was used for all quantitative PCRs. The LightCycler software generated a standard curve from the standards and determined the gene copy number in each test sample. The ratios of gene expression between the target genes (*mexA* & *mexX*) and internal standard (*rpsL*) were expressed relative to those of PAO1 which is set at 1.00[21].

RESULTS

This study was carried out at the Microbiology laboratory of MUHs involving 120 nosocomial *P. aeruginosa* isolates of which 88 isolates exhibited carbapenems resistance phenotypically by the disk diffusion test. A total of 320 different clinical specimens were collected. The highest isolation rate of *P. aeruginosa* was from burn swabs (69/120; 57.5%), blood cultures (21/120; 17.5%) and sputum samples (10/120:8.3%) followed by bronchial aspirates, urine, wound swabs and finally ascetic fluid samples as shown in figure (3)

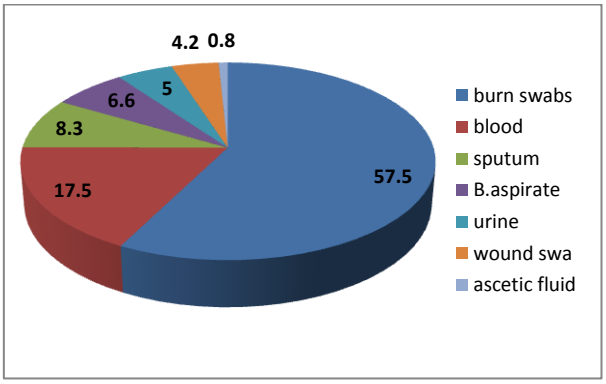

Fig. 3: Distribution of the obtained *P. aeruginosa* isolates among different clinical specimens

Demographic data and risk factors of the studied patients are shown in table (1). Notably, history of carbapenems administration, prolonged duration of hospitalization and/or ICUs stay of more than 14 days, exposure to invasive procedures and associated comorbidities were by far significant risk factors ($P<0.05$) for emergence of MDR *P. aeruginosa* isolates.

Table-1: Relation between carbapenems resistance and demographic data and risk factors of the studied patients

Parameters	*P. aeruginosa* (n=120)				P value
	Carbapenems-resistant (n=88)		Carbapenems- susceptible (n=32)		
	No.	%	No.	%	
Age group (years)					
• **0-5**	15	17.0	2	6.3	>0.05
• **5-20**	17	19.3	5	15.6	
• **20-65**	37	42.1	22	68.7	
• **>65**	19	21.6	3	9.4	
Gender					
• **Male**	52	59.1	19	59.4	>0.05
• **Female**	36	40.9	13	40.6	
Underlying systemic diseases					
• **Yes**	51	57.9	21	65.6	<0.05
• **No**	37	42.1	11	34.4	
Exposure to invasive procedures					
• **Yes**	39	44.3	20	62.5	
• **No**	49	55.7	12	37.5	<0.05
Duration of hospitalization (days)					
• **<7 days**	20	22.7	11	34.4	<0.05
• **7-14 days**	33	37.5	9	28.1	
• **>14days**	35	39.8	12	37.5	
Administration of carbapenems therapy					
• **Yes**	40	45.5	23	71.9	<0.05
• **No**	48	54.5	9	28.1	

P. aeruginosa antibiogram results by disk diffusion method are illustrated in table (2). The highest resistance rates were seen against piperacillin and piperacillin/tazobactam (100% for each) followed by aztreonam (85.5%), cefepime (84.2%), meropenem and imipenem (73.3% for each) and the lowest rates of resistance were seen against tigecycline (17.5%) and colistin (7.5%). Importantly, all (100%) of carbapenems- resistant isolates were considered MDR isolates displaying resistance to one or more members from three or more antibiotic classes.

Table 2: *P. aeruginosa* antibiogram results by disk diffusion method

Antibiotics	Disk content (µg)	*P. aeruginosa* (n = 120)			
		S		R	
		No.	%	No.	%
Piperacillin	100	0	0.0	120	100
Piperacillin/tazobactam	100/10	0	0.0	120	100
Ceftazidime	30	24	20	96	80
Cefepime	30	19	15.8	101	84.2
Aztreonam	30	17	14.2	103	85.8
Amikacin	30	69	57.5	51	42.5
Gentamicin	10	58	48.3	62	51.7
Levofloxacin	5	65	54.2	55	45.8
Norfloxacin	10	61	50.8	59	49.2
Meropenem	10	32	26.7	88	73.3
Imipenem	10	32	26.7	88	73.3
Trimethoprim/sulfamethoxazole	1.25/23.75	29	24.2	91	75.8
Colistin	10	111	92.5	9	7.5
Tigecycline	30	99	82.5	21	17.5
MDR	**92(76.7%)**				
XDR	**17(14.2%)**				
PDR	**11(9.2%)**				

By comparing the MIC results, with and without CCCP, twofold or higher MIC reduction to meropenem was observed in 63/88 (71.6%) isolates on the CCCP-supplemented plates (figure 2) while for CCCP disk synergy test, only 27(30.7%) isolates showed synergy around meropenem disk in the presence of CCCP (figure 1). The RT-PCR assay revealed that, 54 isolates (61.3%) harbored the target genes: 21 isolates (38.9%) were positive for *mexA* alone, 12 isolates (22.2%) were positive for *mexX* alone and 21 isolates (38.9%) were positive for both *mexA* and *mexX* (table 3 & figure 4).

Table 3: MICs, CCCP disk synergy test and RT-PCR results of 88 carbapenems- resistant *P. aeruginosa* isolates

Zone diameters (mm) for CCCP disk synergy test			MIC (mg/L) reduction assay			RT-PCR results		
No. of Isolates	MEM	MEM+CCCP	No. of isolates	MEM	MEM+CCCP	+ve for *mexA* alone	+ve for *mexX* alone	+ve for both *mexA+ mexX*
6	6	12	9	256	32	21	12	21
5	6	15	5	32	4			
1	6	10	4	256	4			
5	7	15	3	32	2			
1	8	12	5	16	8			
3	8	15	3	16	4			
1	6	8	5	32	16			
1	12	15	8	64	4			
1	15	17	3	64	8			
2	6	30	3	64	32			
1	10	15	4	256	16			
			5	256	64			
			6	32	2			
Positive=27(30.7%) Negative= 61 (69.3% without change)			Positive=63 (71.6%) Negative=25 (28.4% without change)			Total PCR positive=54(61.3%) Total PCR negative= 34(38.7%)		

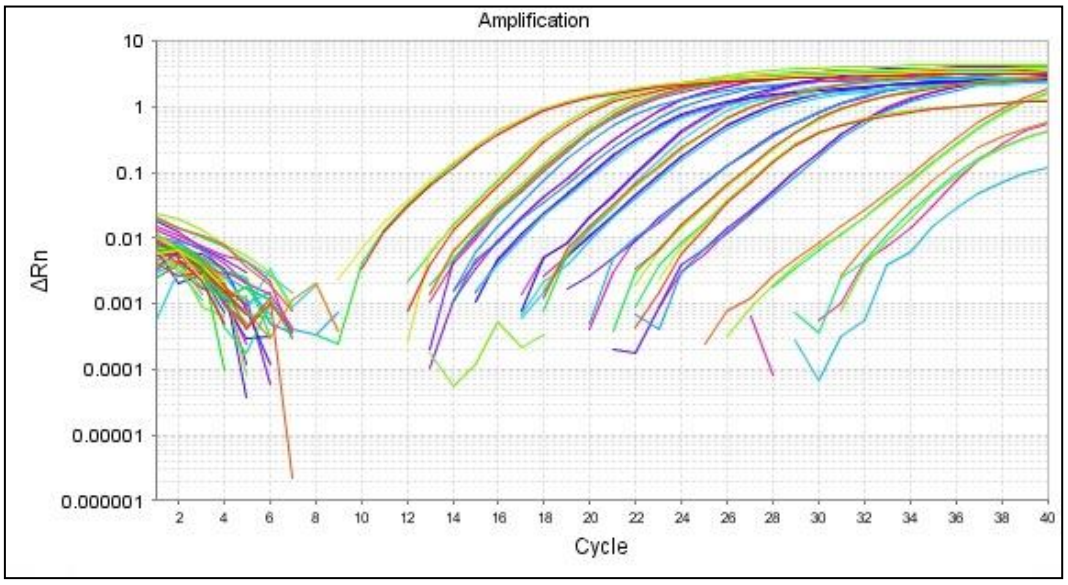

Fig. 4: Time PCR amplification blots of mexA gene (red and green curves) versus rpsL (blue curves for housekeeping gene).

Considering PCR as the gold standard, the sensitivity, specificity and accuracy of CCCP disk test respectively were 46%, 94% and 64.8% while, those for MIC method were 88.9%, 55.9% and 76.1% respectively (table 4).

Table 4: Evaluation of both MICs and CCCP disk synergy tests for demonstration of *P. aeruginosa* efflux pumps overexpression in relation to RT-PCR results

Methods		RT-PCR				Sensitivity	Specificity	Accuracy
		Positive (n=54)		Negative (n=34)				
		No.	%	No.	%			
CCCP disk synergy test (n=88)	+ve (n=27)	25	92.6	2	7.4	46%	94%	64.8%
	-ve (n= 61)	29	47.5	32	52.5			
MICs reduction test	+ve (n=63)	48	76.2	15	23.8	88.9%	55.9%	76.1%
	-ve (n= 25)	6	24	19	76			

DISCUSSION

Carbapenems-resistant *P. aeruginosa* has become an important problem all over the world. These multi-drug resistant clinical isolates represent prominent health threat leaving only few therapeutic options[2].

In the current study, 42% of carbapenems- resistant *P. aeruginosa* isolates were obtained from patients in the age group of 20- 65 years old. A similar observation was also documented by *Srinivas et al.*[22] who reported that, isolation of MDR *P. aeruginosa* was more frequent in the age group of 22- 66 years (66.67%). As for underlying health impairment and associated co-morbidities, the current study revealed that, the prevalence of carbapenems- resistant isolates was significantly superior in patients complaining associated co-morbidities (57.9%) compared to those with no underlying systemic diseases. Patients infected with carbapenems–resistant *P. aeruginosa*, had one of the following underlying medical conditions; malignancy, chronic renal impairment, chronic liver disease and diabetes. This finding was in agreement with *Ho et al.*[23] and *Tumbarello et al*.[24]who addressed severe underlying medical impairment as one of the risk factors for emergence of carbapenems-resistant isolates.

Regarding invasive procedures, 44.3% of *P. aeruginosa*- infected patients who developed carbapenems resistance were subjected to invasive techniques such as mechanical ventilation, urinary catheterization and CVL (central venous line). This observation came in accordance with *Lucet*[25] and *Christian et al.*[26] who stated that, exposure to invasive devices is considered potential factor for carbapenems resistance.

As regards, duration of hospital stay, we observed that, the highest rate of isolation of carbapenems-resistant *P. aeruginosa* was from patients with duration of hospital stay more than two weeks (39.8%), followed by duration of 7-14 days and finally hospital stay of less than 7 days which agreed with Graffunder *et al.*[27] and *Tumbarello et al.*[24] .Possible explanation of that observation is that, prolonged hospital stay favors the transmission of nosocomial infections.[26]

Importantly, 45.5% of patients infected with carbapenems- resistant isolates had history of carbapenems intake which was in agreement with *Guilherme et al.*[28] and *Carmeli et al.*[29]. The authors declared that, broad-spectrum antibiotic provoke the eradication of competitive microbiota and select for strains with overexpression of efflux pumps.

According to the present results, the obtained *P. aeruginosa* isolates exhibited high antibiotic resistance rates reaching up to 100% for piperacillin and piperacillin/tazobactam, 85.8% for aztreonam, 84.2% for cefepime, 80% for ceftazidime, 73.3% for both meropenem and imipenem, 51.7% for gentamicin, 49.2% for norfloxacin, 45.8% for levofloxacin and finally 42.5% for amikacin which are commonly used to treat *P. aeruginosa* infections. Notably, 76.7%, 14.2% and 9.2% of the isolates respectively were MDR, XDR and PDR isolates.

On the other hand, the lowest resistance rates were for tigecycline (17.5%) and colistin (7.5%). *Taher et al.*[30] also reported high resistance rates to imipenem, cefepime, ciprofloxacin, and gentamicin; all their isolates were resistant to three or more antibiotics tested except for colistin, which showed the highest antibacterial activity (100%). Similarly, *Abass et al.*[31] found all *P. aeruginosa* isolates to be highly resistant to amoxicillin/clavulanic, sulphamethoxazole/ trimethoprim, and ceftazidime (100% for each), cefotaxime (92%), ceftriaxone and cefepime (74% for each) and that all of *P. aeruginosa* isolates were considered MDR. In the same field, *Mohamed et al.*[32] and *El-Mahdy* and *El-Kannishy*[33] reported more than 70% of *P. aeruginosa* isolates as MDR isolates.

Interestingly, we observed resistance to unrelated antibiotic classes; most of *P. aeruginosa* isolates exhibited multidrug resistance to two or three tested antibiotic classes. It could be assumed that, part of these multidrug cross-resistances among *P. aeruginosa* are caused by overexpression of multidrug efflux pumps and every efflux pump expels several antibiotic classes. Ultimately, improper use of antibiotics could cause resistance to other classes by triggering the overexpression of efflux pumps and select for mutants with multidrug cross-resistance[18].

The current study applied combined phenotypic and genotypic approaches for diagnosis of carbapenems resistance mediated by the two MexAB-OprM and MexXY-OprM efflux pumps in *P. aeruginosa*. Phenotypically, we used the broad-spectrum efflux pump inhibitor compound, CCCP, and investigated the efflux pump overexpression using two methods; CCCP disk synergy and MIC reduction tests. However and as already noted by others[34,35], interpretation of phenotypic data remains difficult with clinical strains, probably because of the co-expression of resistance mechanisms other than efflux. Those two phenotypic methods devised here provides a first level of differentiation among the causes of carbapenems resistance as high resistance of *P. aeruginosa* may be attributed to several mechanisms including efflux pumps, reduced activity of outer membrane porins and production of AmpC β-lactamases[36].

In our study, active efflux was detected in 27 (30.7%) out of 88 *P. aeruginosa* isolates by CCCP disk synergy test and in 63 isolates (71.6%) by MIC reduction test. This was in agreement with *Taher et al.*[30]who found 30 out of 77 *P. aeruginosa* isolates to express efflux pump by CCCP disk test and 59 out of 77 by MIC CCCP reduction assay. The synergy observed between CCCP and meropenem suggests its selective extrusion by the efflux systems. However, although meropenem is recognized and ejected by the up-regulated efflux pumps, a mutation in OprD protein is also deemed necessary for achieve resistance. It is presumed that porins mutations are probably also the reason for the isolates exhibiting intermediate or low-level resistance to carbapenems which is not affected by efflux pumps. Therefore, the absence of synergy between CCCP and meropenem disks in some resistant isolates suggests the presence of alternative resistance mechanisms such as outer membrane porins gene mutations[37,38].

In the present study, RT–PCR for the overexpression of *mexA* and *mexX* genes was done. 21/54 (38.8%) isolates were positive for *mexA* alone, 12/54 (22.2%) isolates were positive for *mexX* alone and 21/54 (38.8%) isolates revealed co-existence of both *mexA* and *mexX* genes. Higher result were reported by *Abbas et al*[31] who detected active efflux in all isolates, similarly, *Rana et al.*[38] and *Al-Grawi et al.*[39] reported active efflux in all MDR isolates.

In agreement with *Mesaros et al.*[40] basal expression level of *mexX* in the present study, was much lower than that of *mexA* but both efflux pumps were overexpressed 4 to 8 times in resistant strains, and some clinical isolates were found to express both efflux pumps at the same time and this explain the cause of multidrug resistance pattern revealed in our study.

The performance of the phenotypic methods in relation to RT-PCR as a gold standard was also evaluated. It clearly appears that the sensitivity, specificity and accuracy of CCCP disk synergy test respectively were 46%, 94% and 64.8% while, those for MIC method were 88.9%, 55.9% and 76.1%. CCCP disk synergy test has high specificity, while MIC CCCP reduction test is a good sensitive test. Our possible explanation is that some of the efflux pumps are inactive now and could be overexpressed later.

CONCLUSION

Emergence of multidrug resistance due to carbapenems misuse is alarming. Further studies on efflux pump appear to be an attractive approach for improving the clinical efficacies of antibiotics that are substrates of these pumps. This knowledge may be helpful, at the level of the individual patient, for rationalizing the antibiotic choice and at the hospital level, for defining antibiotic policies, based on epidemiological surveys evidencing the most prevailing resistance mechanisms.

Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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