



Microbes and Infectious Diseases

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

Original article

Expression of *MexAB-OprM* efflux pump system and meropenem resistance in *Pseudomonas aeruginosa* isolated from surgical intensive care unit

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ARTICLE INFO

Article history:

Received 5 August 2021

Received in revised form 18 September 2021

Accepted 21 September 2021

Keywords:

Meropenem

Pseudomonas aeruginosa

Resistance

Efflux pump

ABSTRACT

Background: Meropenem resistance of *Pseudomonas aeruginosa* (*P. aeruginosa*) is considered an increasing problem. Efflux pump is one of multiple mechanisms that are responsible for this resistance. This study aimed to phenotypically and genotypically detect prevalence of efflux pump mediated meropenem resistance among *P. aeruginosa* isolates. **Methods:** *Pseudomonas aeruginosa* was isolated from different clinical specimens and identified by conventional methods and confirmed by Viètek MS Maldi-TOF Mass Spectroscopy. Antibiotic susceptibility test was done by disc diffusion method then minimum inhibitory concentrations (MIC) for meropenem was detected twice by agar dilution method without and after addition of carbonyl cyanide m-chlorophenylhydrazone (CCCP). Efflux pump genes were detected by polymerase chain reaction (PCR). **Results:** Out of 265 specimens, 78 *P. aeruginosa* were isolated with an isolation rate (29.4%). By disc diffusion method and MIC by agar dilution methods, 35 (44.8%) isolates were meropenem resistant. There was a significant difference regarding distribution of efflux pump genes in meropenem resistant isolates as 23 isolates (65.7%) were positive for efflux pump genes and 12 (34.3%) were negative (p value= 0.13). The MICs of meropenem for *P. aeruginosa* isolates were significantly decreased after addition of CCCP where MIC of 21 (60%) meropenem resistant isolates had an efflux pump-overexpressing phenotype (p value =0.001). **Conclusion:** High prevalence of meropenem resistance in *P. aeruginosa* is mediated by efflux pump genes including, *mex A*, *mex B* and *opr M*.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the important pathogens reported in community and healthcare-associated infections (HAIs). Emergence of pan drug-resistant (PDR) and multidrug-resistant strains (MDR) is considered a significant public health issue not only impairing the

antibacterial agents' effectiveness but also wasting the efforts for developing new drugs [1].

The resistant phenotypes are attributed to the interaction between the poly specific multidrug efflux pumps and the low permeability of outer membrane which may be of intrinsic origin or via

DOI: 10.21608/MID.2021.92720.1187

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acquiring genes encoding resistance determinants [2,3].

Structural genes for at least 12 resistance-nodulation-cell division (RND) type efflux systems had been detected such as *Mex AB-OprM*, *Mex CD-OprJ*, *Mex EF-OprN* and *MexXY-OprM* where the *MexAB-OprM* is the most expressed multidrug efflux pump in *P. aeruginosa* conferring resistance to a wide range of antimicrobials [4, 5].

Carbapenems are very important for the management of infections due to *P. aeruginosa*. The interaction of efflux pumps with meropenem differs from that with imipenem. Both are able to enter the cell via the *OprD* pathway, only meropenem is a substrate of the *MexAB-OprM* efflux pump [3].

Efflux pump inhibition can be produced by interference with either the regulator of the efflux pump expression or disturbance of the efflux pump components assembly or interference with the energy required for the pump activity. Moreover, blocking of outer pores causing antibiotics efflux [6].

Efflux pump inhibitors (EPIs) play an important role in success of therapy and decrease the level of resistance and increase the intracellular concentration of the therapeutic drugs. Their toxic effect is the main issue challenged [7].

Carbonyl cyanide *m* chlorophenylhydrazone (CCCP) was used as one of the EPIs for *P. aeruginosa* infections by its oxidative phosphorylation action which reduces the ATP production and increases the bacterial membrane permeability by interfering with proton motive force [8].

Hence this study was conducted to investigate the rate of meropenem resistance that was attributed to efflux-pump *Mex AB-OprM* genes expression in *P. aeruginosa* isolated from patients admitted at surgical ICU and subsequently, helping to improve their management and outcome.

Material and Methods

Study design and participants

This cross-sectional observational study was executed over one year in surgical ICU and Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University. The study included patients admitted to surgical ICU during study period if they developed fever, leukocytosis and other evidences of infections.

Ethical approval

The study was approved by Zagazig University Institution Review Board (ZU-IRB) (Approval code 6825). This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from patients or their relatives.

Microbiological work up

Clinical samples collection and *P. aeruginosa* identification

A total of 265 clinical samples were aseptically collected according to the site of infection from ICU admitted patients. Samples included endotracheal aspirates, urine, pus, and blood. Detailed history of patients included age, sex, length of hospital stays, use of invasive medical devices and previous administration of empirical antibiotics were stated. Samples were transported and processed for isolation and identification of *P. aeruginosa*. Initial identification was done via conventional biochemical methods [9] and confirmed to the species level by Viètek MS Malditof Mass Spectroscopy (Biomerieux. Inc. Durham.USA).

Antimicrobial susceptibility of isolates

Antimicrobial susceptibility test was performed according to the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines [10]

- Kirby–Bauer disk diffusion method: Piperacillin (100ug), piperacillin-tazobactam (100/10µg), aztreonam (10ug), amikacin (30µg), ceftazidime (30 µg), cefepime (30µg), ciprofloxacin (5 µg), levofloxacin (5 µg), imipenem (10 µg) and meropenem (10 µg) (Oxoid, UK) were used. Multi- drug resistant *P. aeruginosa* (MDR-PA) was defined when resistance to more than one antibiotic in 3 or more groups of antimicrobials was recorded [11].
- Minimal inhibitory concentration (MIC) by agar dilution method for meropenem ranged from 0.125 to 512 mg/L. According to the established breakpoint values recommended by CLSI, the *P. aeruginosa* isolates with MIC \geq 8mg/L are considered as meropenem resistant. *Pseudomonas aeruginosa* ATCC®27853 was used as a quality control strain (American Type Culture Collection [ATCC], Manassas, VA, USA).

Phenotypic detection of efflux pump by Carbonyl cyanide m-chlorophenylhydrazone (CCCP) test

The isolates that show resistance to meropenem were subjected to phenotypic detection of efflux pump by a repeating agar dilution test after addition of CCCP to Mueller-Hinton agar with a final concentration of it at 2.5ug/ml and then MIC was estimated. An efflux pump-overexpressing phenotype was defined as any strain exhibiting at least a fourfold decrease in MIC when tested after CCCP addition [1,12].

Genotypic detection of genes encoding efflux pump mediated meropenem resistance by PCR

All *P. aeruginosa* isolates were tested by PCR for the presence of 3 genes which were: *mex A*, *mex B* and *oprM* genes. DNA was extracted using the QIAmp DNA Mini Kit (QIAGEN, Germany)

according to the manufacturer's instructions, and stored at -20°C until amplification was performed. PCR was performed using thermal cycler (Veriti®96-Well Thermal Cycler, Applied Biosystems, Singapore). Polymerase chain reaction was performed in a final volume of 20 µl including, 1 µl of the template DNA, 1 µl of each of the oligonucleotide primers and 13 µl of sterile deionized water were added to each PCR bead of Maxime PCR PreMix Beads (iNtron, Certified Company, Germany). Primers and conditions were listed below in tables (1) and (2). The PCR products were analyzed by agarose gel electrophoresis on 1.5% agarose (w/vol.) containing 0.5-mg/mL ethidium bromide (Qiagen, Germany), using a 100-bp DNA ladder as the size marker (Roche, Germany).

Table 1. Primer sequence of *mexA*, *mexB* and *oprM* genes [3].

Gene	Primers	Base Pair
<i>Mex A</i>	F CTACGAGGCCGACTACCAGA R TGCAGGCCTTCGGTAATGAT	722
<i>Mex B</i>	F CCGTGAATCCCGACCTGATG R TGACATGATGGCTTCCGCAT	255
<i>Opr M</i>	F TACCAGAAGAGTTTCGACCTGAC R CATGTGTCAAAACAGTCACCTCC	812

Table 2. Conditions of PCR cycles [13].

Cycle	Temperature	time	Number of cycles
Initial Denaturation	94 °C	5 min	1
Denaturation	94 °C	30 sec	35
Annealing	55 °C	30 sec	35
Extension	72 °C	1.5min	35
Final Extension	72 °C	7 min	1

Statistical analysis

Statistical packages (EPI-info Version 6.04 and SPSS Version 20 inc. Chicago, USA) were used to analyze collected data. Chi-square test was used to compare proportions. A *p*-value<0.05 was considered to be statistically significant at 95% confidence interval [14].

Results

Out of 265 specimens 78 *P. aeruginosa* were isolated with isolation rate (29.4%) mainly from endotracheal aspirate (44%) followed by urine (36%) pus (18%) and blood (2%).

Based on the results of AST by disc diffusion method, more than two thirds of isolates (68%) were MDR and showed high resistance to piperacillin (75%) and aztreonam (72%). Moreover,

35 (44.8%) were meropenem resistant. Antibiotic resistance patterns have been presented in **figure (1)**. Out of 35 meropenem resistant isolates, 23 isolates (65.7%) were positive for efflux pump genes where *Mex A* gene was detected in 19 (54.2%) while *Mex B* was detected in 18 isolates (51.4%) and both genes were detected in 14 isolates (40%) while *Opr M* was detected in 3 isolates (8.6%) and this was statistically significant ($p= 0.013^*$) as shown in **table (4)**. Preliminary results of meropenem susceptibility test using the disk diffusion method, showed that isolates (44.8%) were resistant and they were confirmed by MIC detection and these isolates were resistant to meropenem ($MIC \geq 8$ mg/L). By using CCCP as EPI, the MIC of 21 (60%) of the

meropenem resistant isolate had an efflux pump-overexpressing phenotype (4-fold decrease or more in MIC) on the CCCP-supplemented plate, MIC of 7 (20%) isolates decreased 2 fold and MIC of last 7 (20%) isolates not changed after CCCP addition and this was demonstrated in **table (5)**. Regarding agreement between phenotypic and genotypic methods, 21 (91.3%) of *P. aeruginosa* isolates carrying efflux pump genes had an efflux pump-overexpressing phenotype, 2 (8.7%) isolate were carrying efflux pump genes but were phenotypically negative and lastly efflux pump was not detected either phenotypically or genotypically in 7 isolates and this was statistically significant ($p= <0.001^{**}$) as shown in **table (6)**.

Table 3. Distribution of isolated *P.aeruginosa* according to type of specimen.

Infection	No. of samples	No. and % of isolates		X ²	p value
		No.	%		
Endotracheal aspirate	94	34	44.0	42.324	<0.001**
Urine	82	28	36.0		
Pus	70	14	18.0		
Blood	19	2	2.0		
Total	265	78	100.0		

**= highly significant

X²=Chi-square test

Table 4. Frequency of efflux pump genes among meropenem resistant isolates.

Gene	No of isolates positive		X ²	p value
	No.	%		
Efflux pump genes positive	23	65.7	12.5	0.013*
<i>Mex A</i>	19	54.2		
<i>Mex B</i>	18	51.4		
<i>Opr M</i>	3	8.6		
<i>Mex A and Mex B</i>	14	40.0		
<i>Mex A, Mex B and Opr M</i>	3	8.6		
Efflux pump genes negative	12	34.3		
Total	35	100.0		

* = significant

X²=Chi-square test

Table 5. Antibiotic susceptibility by agar dilution after CCCP addition.

Interpretation of MIC test after addition of CCCP	Isolates		X ² test	p value
	No.	%		
An efflux pump-overexpressing phenotype (4-fold decrease or more)	21	60.0	16.8	<0.001**
Negative test (2-fold decrease)	7	20.0		
Negative test (No change in MIC)	7	20.0		
Total	35	100.0		

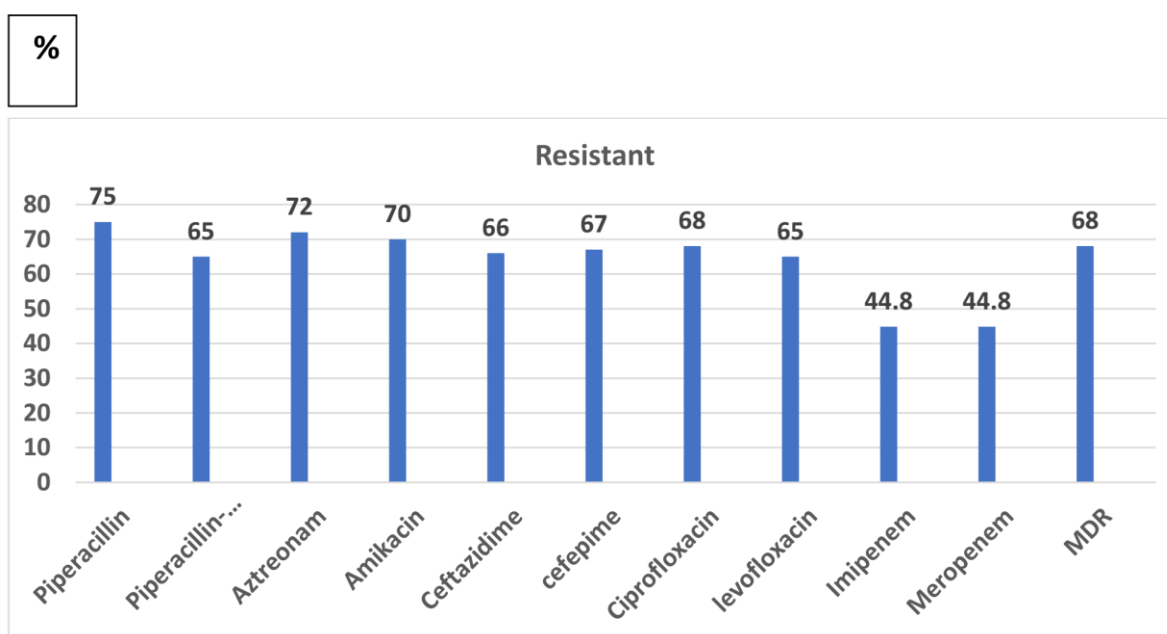
**= highly significant

X²=Chi-square test.

Table 6. Agreement between phenotypic and genotypic methods.

Interpretation of MIC test after addition of CCCP (phenotypic)	Genotypic				X ² test	p value
	Negative No=12		Positive No= 23			
	No.	%	No.	%		
An efflux pump-overexpressing phenotype (21)	0	0.0	21	91.3	20.628	<0.001**
Negative test (2-fold decrease) (7)	5	41.7	2	8.7		
Negative test (No change in MIC) (7)	7	58.3	0	0.0		

**= highly significant

X²=Chi-square test**Figure 1.** antibiotic resistance by disc diffusion method.

Discussion

Treatment of *P. aeruginosa* infections is difficult not only due to their intrinsic resistance to many antimicrobials but also, their ability to easily acquire antibiotic resistance. Carbapenems are considered corner stone treatment option for these infections. However, increased Carbapenems resistance in *P. aeruginosa* isolates has been reported and it ranged from 10% to 50% in most countries [15]

In this study, 78 *P. aeruginosa* were isolated out of 265 specimens with isolation rate (29.4%). They were distributed as; 44%, 36%, 18% and 2% from endotracheal aspirate, urine, pus and blood, respectively. This was in agreement with another study done by **Mohamed et al.**[16] to detect prevalence of *P. aeruginosa* isolated from patients

admitted at Zagazig University and Al-Ahrar hospitals reporting 33.3% isolation rate mainly from endotracheal aspirate (29%) followed by urine (27%), burn (25%) and others (19%). in a similar study, an isolation rate (20.7%) was detected in Cairo University hospitals where most of isolates were from lower respiratory tract infections (44.2%) followed by surgical site infections (37.5%) and others by **Wassef et al.** [17]. A lower frequency (11.3%) of *P. aeruginosa* was reported in a cross-sectional study on *P. aeruginosa* having metallo-beta-lactamase isolated from patients at Alexandria University Students' Hospital by **Abaza et al.** [18].

Multi- drug resistant *P. aeruginosa* are increasingly isolated from nosocomial infections so, the susceptibility of these isolates to commonly used antibiotics should be continuously investigated to be

a guide for proper selection of antimicrobials for management of these infections [19].

Regarding antibiotic susceptibility of isolated *P. aeruginosa*, an increased antimicrobial resistance rate was observed as more than two thirds (68%) of isolates were MDR. This was similar to results of **El-sayed and Fahmy** [20] in their study on biofilm formation by *P. aeruginosa* had found that prevalence of MDR were (69.1%) isolates. Also, **Abdel-Rhman and Rizk** [19] in their study on *P. aeruginosa* isolated from patients with urinary tract infections at Mansoura, Egypt found that (51.3%) isolates were MDR. **Fotouh et al.** [21] concluded decreased *P. aeruginosa* susceptibility to antimicrobials and reported that (76 %) of *P. aeruginosa* isolated from surgical site infection of patients admitted at Zagazig University hospitals were MDR. on the other hand, a lower resistance rate was detected by **Hassuna et al.** [22] in their study in Minia which isolate *P. aeruginosa* from ventilator associated pneumonia patients and showed that MDR frequency was 22.5%. Discrepancy in results of antibiotic susceptibility in different studies was attributed to different infection and specimen type, patients risk factors, site of admission of patient and implementation of infection control measures.

Concerning meropenem susceptibility in this study, 35 (44.8%) of isolates were meropenem resistant. In a similar study in Egypt by **Basha and coworkers** [23], it was reported that 57% of their isolated *P. aeruginosa* were resistant to meropenem. Also 62% of isolates from health care associated infections at Suez Canal University Hospitals were meropenem resistant **Kishk et al.** [1]. **Negm et al.** [24] had found higher rate of resistance as about 84.7% of their isolates from different ICUs in Zagazig university hospitals were resistant to meropenem. In China 64.7% of *P. aeruginosa* isolated from different clinical specimens were meropenem resistant (**Wang and his colleagues** [25]). However, a lower resistance rate was detected by **Fotouh et al.** [21] who reported that (22.2%) of *P. aeruginosa* isolated from surgical site infection of patients admitted at Zagazig University hospitals were meropenem resistant. High meropenem resistance rate detected might be correlated with the selective pressure from increased use of carbapenems for *P. aeruginosa* infections [23].

There was a significant difference regarding distribution of efflux pump genes in meropenem resistant isolates as 23 isolates (65.7%)

were positive for efflux pump genes and 12 (34.3%) were negative confirming that efflux pump had an important role in conferring meropenem resistance in *P. aeruginosa*. *Mex A* gene was detected in 19 (54.2%) while *Mex B* was detected in 18 isolates (51.4%) and both genes were detected in 14 isolates (40%) while *Opr M* was detected in 3 isolates (8.6%). This was in agreement with **Pourakbari et al.** [26] who reported also that overexpression of *MexA* gene was observed in 25 isolates (55.5%), *MexB* in 24 isolates (53.3%) and *OprM* in 16 isolates (35.5%) and **Murugan et al.** [13] detected that frequency of efflux pump genes was as follow, (51%) *MexA*, (46.5%) *MexB*, and (40.5%) *Opr M*. In addition, overexpression of efflux pumps was detected by **Abbas et al.** [27] in their study on *P. aeruginosa* isolated from urinary tract infections and had found that 100% of isolates were carrying the 3 genes and that efflux system contributes to the natural bacterial resistance to a wide range of antibiotics and detergents. Also, **Rana et al.** [28] reported presence of active efflux in all MDR isolates. Similarly, a cross sectional study in Suez Canal University Hospital by **Kishk et al.** [1] found that overexpression of efflux pumps could be the leading cause of MDR in bacteria and that all their isolated PDR *P. aeruginosa* were carrying both *mex A* and *mex B* genes. While, *Mex A* gene was detected in 88.2% (120 strains), *Mex B* was detected in 70.5% (96 strains) and both genes were detected in 80 strains (58.8%). Also, **Tomas et al.** [29] concluded that increased *mex A* expression was the main mechanism of resistance to meropenem ($p = 0.027$). On the other hand, **Ozer et al.** [30] reported that no significant relation was found between *mex A* gene carriage and meropenem resistance in *P. aeruginosa* isolated from lower respiratory tract infections in intensive care unit in Turkey. This could be explained by that meropenem resistance was mediated by another mechanism other than efflux pump.

By using CCCP as EPI, a significant reduction in MIC on addition CCCP proved the role of efflux pumps in meropenem resistance. In this study, 21 (60%) of meropenem resistant isolates showed a positive efflux pump overexpressing-phenotype while other 14 isolates were negative where MIC of 7 (20%) isolates decreased 2-fold and MIC of last 7 (20%) isolates not changed after CCCP addition. This was in accordance with **Kishk et al.** [1] and **Khalek et al.** [3] who found that the main mechanism to carbapenems resistance was via

efflux pump activation which was confirmed phenotypically by CCCP addition as a pump inhibitor. This was also similar to reports by **Choudhury et al.** [31] who had detected efflux pump activity phenotypically and marked decrease in MIC in all their isolates when CCCP was added.

Regarding agreement between phenotypic and genotypic methods, 21 (91.3%) of *P. aeruginosa* isolates carrying efflux pump genes had an efflux pump-overexpressing phenotype, in this study. **Khalek et al.** [3] agreed with our results and reported 100% correlation between phenotypic and genotypic detection. In addition, **Pan et al.** [32] concluded that *MexAB-OprM* efflux pump was detected in 76.9 % of efflux phenotype-positive strain. Moreover, **Choudhury et al.** [31] in India found that 22 *p. aeruginosa* isolates were found to possess efflux pump activity phenotypically and that *MexAB-OprM* efflux pump was found to be overexpressed in all these isolates. On the other hand, a lower frequency was detected by **Taher et al.** [33] who found that all their *p. aeruginosa* were carrying *mex A* gene but only about half of these isolates had an efflux pump-overexpressing phenotype and they attributed that to inactive efflux pump which might be overexpressed later.

Conclusion

High prevalence of multidrug- resistant and meropenem resistant *P. aeruginosa* was detected and efflux pump system (*Mex AB - opr M system*) was the main mechanism of meropenem resistance in these isolates.

Conflict of interest

All authors declare no conflict of interest in this work.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Contributors and authorship

All authors have made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content. Finally, they have approved the version to be submitted.

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