

ORIGINAL ARTICLE

Phenotypic and Genotypic Detection of Efflux Pump Mediated Meropenem Resistance in *Pseudomonas aeruginosa* isolates from Catheter Associated Urinary Tract Infection

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ABSTRACT

Key words:

Efflux pump, meropenem, CAUTI

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Background: Catheter associated urinary tract infection is considered as the commonest complication of indwelling catheter. Chronic indwelling catheters are an important reservoir of different multiresistant gram-negative organisms as *Pseudomonas aeruginosa*. **Methodology:** Our study was carried out on patient with CAUTI who were admitted to Urology Department at Tanta University Hospital. *Pseudomonas aeruginosa* isolates that were resistant to meropenem were subjected to phenotypic detection of efflux pump mechanism by CCCP test and genotypic detection of efflux pump gene by PCR. **Results:** *P. aeruginosa* was isolated from 14.6% of CAUTI. Meropenem resistance was found in 19.6% of isolates. We found 100% correlation between phenotypic and genotypic detection method as regard mex A gene.

INTRODUCTION

Urinary Tract Infection (UTI) is one of the commonest infections all over the world. It is considered as the 2nd commonest infection of the body.¹

Several risk factors predispose to urinary tract infection. Catheterization is the commonest risk factor for its occurrence.² Catheter Associated Urinary tract infection (CAUTI) is responsible for about 40% of nosocomial UTI.³ Instillation of catheter may cause damage of mucosal layer, which disrupts the natural barrier and predispose to colonization of microorganisms⁴

Several microorganisms are responsible for UTI as *Escherichia coli* (*E. coli*), *Klebsiella* species, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Acinetobacter*⁵

Recently, the antibiotics abuse and random antibiotics treatment of patients with UTI, lead to development of resistant strains of microorganism⁶

P. aeruginosa is an opportunistic human pathogen that infect immunosuppressed patients, it is one of the commonest causes of CAUTI⁷ *P. aeruginosa* possess several virulence factors including phenazines, quorum sensing, biofilm formation and siderophores⁸

Quorum Sensing (QS) is one of the virulence factors of *Pseudomonas aeruginosa*: it is a cell-to-cell communication phenomenon through small diffusible signals called autoinducers. It is used by bacteria to monitor their surrounding environment and to control gene expression depending on their demands⁹

Siderophores are iron chelating agent that are produced by *Pseudomonas aeruginosa* in form of

pyocyanins and pyoverdines. They enable the bacteria to survive in the urine which is an iron poor environment^{10,11}

Pseudomonas aeruginosa produces redox-active pigments called phenazines that greatly affect gene expression and metabolic flux. It was found that phenazines may play a role in antibiotics resistance to *Pseudomonas aeruginosa*¹² Several phenotypic methods for typing *P. aeruginosa* are available, as biotyping, bacteriocin typing and antibiotic typing. However *p. aeruginosa* are phenotypically unstable so genotyping method is more reliable.^{13,14}

Virulent *Pseudomonas aeruginosa* harbors many virulence genes as *exoS*, *exoT*, *exoU*, and *exoY* genes that are expressed into protein products related to type III secretion systems (TTSS). These products are cytotoxic¹⁵

Pseudomonas aeruginosa is known by its intrinsic resistance to antibiotics and for its ability to acquire genes encoding resistance determinants. Carbapenems have antipseudomonal activity, so they are important agents for the therapy of infections due to *P. aeruginosa*. The development of carbapenem resistance among *P. aeruginosa* strains is multifactorial. It may be via integron or plasmid transmission of gene encoding for carbapenem resistance, increase efflux system expression or decrease porin expression. The interaction of efflux pumps with meropenem differs from that with imipenem. While it is believed that both meropenem and imipenem are able to enter the cell via the OprD pathway, only meropenem is a substrate of the MexAB-OprM efflux pump^{16,17,18}

The MexAB-OprM efflux pump belongs to the superfamily of ribonucleoproteins and consists of an inner membrane (MexB), a periplasmic membrane fusion protein (MexA) and a channel-forming outer membrane protein, OprM¹⁹

Our study aimed to detect the presence of efflux pump mediated meropenem resistance in isolates of *Pseudomonas aeruginosa* from patients with catheter associated urinary tract infection (CAUTI) phenotypically and genotypically.

METHODOLOGY

Subjects and design:

This study was carried out during the period from March 2018 to March 2019. A total of 350 urine samples were collected from patient admitted to hospital at both Urology Department and Intensive Care Units. All patients suffered from urinary tract infection and all patients were catheterized.

Inclusion criteria:

Catheterized admitted patients with UTI

Exclusion criteria:

Patients with UTI without catheter

Ethical approval for this study was provided by ethical and research committee.

Bacterial strains:

All urine specimens were obtained through the catheter port using aseptic technique or by puncturing the catheter tubing with a sterile syringe in patients or during catheter replacement by collecting fresh urine sample from the freshly placed catheter. Urine collection from urine bag was prohibited.

All urine samples were collected in sterile containers and transported to the Microbiology Laboratory.

All urine samples were cultured on nutrient agar, blood agar and MacConkey at 37 °C for 24 hours aerobically. All cultures that show Colonies that produced pyoverdinin, pyocyanin, and pyorubin pigments were subcultured on nutrient agar. *Pseudomonas aeruginosa* isolates were identified by colonial morphology, microscopic examination, motility and conventional biochemical reactions: oxidase test, catalase test citrate test, gelatinase liquefaction and nitrate reduction.

Antimicrobial susceptibility testing:

P-aeruginosa isolates were subjected to:

Antibiogram using the following antibiotics discs norfloxacin (30 µg/disk), imipenem (30 u/disk), gentamycin (10 µg/disk), ciprofloxacin (5 µg/disk), cefipime (30 µg/disk), cefoperazone (30 µg/disk), meropenem (10 µg/disk), amikacin (30 u/disk), ceftazidime (30 µg/disk), aztreonam (30 µg/disk) and chloramphenicol (30ug). (Oxoid UK).

Phenotypic detection of efflux pump by CCCP test:

The isolates that show resistance to meropenem were subjected to phenotypic detection of efflux pump

using Carbonyl Cyanide M-Chlorophenyl Hydrazone test (CCCP Test) as follow²⁰:

Principle of test: It is a phenotypic test for detection of over expression of efflux system as a main mechanism for resistance to meropenem. It depends on inhibition of efflux pump by chemical method and subsequent inhibition of resistance to meropenem.

Muller Hinton Agar was prepared twice, one time with adding CCCP as a efflux pump inhibitor and another time without its addition. The isolates that showed meropenem resistance by disc diffusion method were inoculated on Muller Hinton Agar with and without cccp. The size of inhibition zones were compared. Test is considered positive if the inhibition zone of meropenem is wider in the agar plate with CCCP than the one in the plate without pump inhibitor.

Measurement of MIC of meropenem before and after addition of CCCP:

It was measured using agar dilution method according to Clinical and Laboratory Standard Institute (CLSI) Guidelines. Final concentration of CCCP at 2.5ug/ml was added to MHA then 10ug /ml of meropenem was added to obtain final concentration of 0.12 to 512 ug/ml. *Pseudomonase* control strain ATCC27853 was used.

Detection of genes encoding efflux pump mediated meropenem resistance by PCR:

All pseudomonas 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 GmbH, Hilden, Germany), according to the manufacturer's recommended procedures.

The DNA was eluted in 100 µL of elution buffer (Qiagen) and stored at -20 °C until use.

PCR targeting these genes were performed using primers specific to each gene (table 1).

Table 1: Primer sequence of mexA, mexB and oprM genes.

Gene	Primers	Base Pair
mex A	F CTACGAGGCCGACTACCAGA R TGCAGGCCTTCGGTAATGAT	722
Mex B	F CCGTGAATCCCGACCTGATG R TGACATGATGGCTTCCGCAT	255
oprM	F TACCAGAAGAGTTTCGACCTGAC R CATGTGTCAAACAGTCACCTCC	812

RESULTS

This study was carried out on 350 urine samples collected from catheter from patients at ICU and Urology Department of Tanta University Hospital, who were suffering from CAUTI.

Antibiotic susceptibility:

Pseudomonas aeruginosa was isolated from 51 out of 350 (14.6%) urine samples.

All isolates of *Pseudomonas aeruginosa* were resistant to cephoperazone. Most isolates 82.5% were resistant to chloramphenicol. While 25% were resistant to amikacin, 35% to aztreonam, 50% to gentamycin, 11% to ciprofloxacin, 8% to norfloxacin, 33% to ceftazidime and 28% to cefepime.

In our study 10 isolates of *Pseudomonas aeruginosa* were resistant to meropenem (19.6%) while 41 isolates were meropenem sensitive.

Phenotypic and genotypic detection of efflux pump mediated meropenem resistance:

9 out of 10 isolates of *Pseudomonas aeruginosa* showed positive results during CCCP test. We found that these 6 phenotypically resistant isolates by disc diffusion method showed sensitivity to meropenem after addition of efflux pump inhibitors (EPIs).

There were obvious increase in the diameter of the zone of inhibition by >7mm. This confirmed that meropenem resistance among these 6 isolates was mediated via efflux pump mechanism. (Fig. 1)

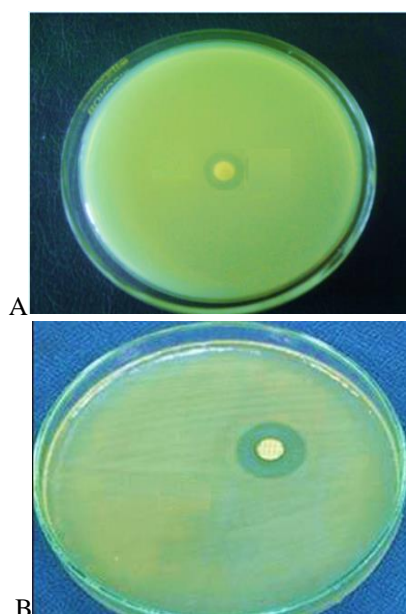


Fig 1: Muller Hinton Agar without CCCP (A) and after CCCP (B)

PCR assay for 10 isolates indicated the presence of mex A in all 6 positive CCCP test isolates (fig 2). mex A gene was not detected in any of other 4 isolates. mex

B gene was detected in 4 isolates out of 6 isolates. It was not found in any of 4 isolates.

Opr M gene was detected only in one out of 10 isolates.(table2)

We found 100% correlation between phenotypic and genotypic detection methods as regard mex A gene.

Table 2: Result of genotypic test

Isolate no	1	2	3	4	5	6
mex A	+	+	+	+	+	+
mex B	+	+	+	+	-	-
oprM	+	-	-	-	-	-



Fig2: Positive lane 1 ,3 ,4 ,7 8 and 10 for mexA gene (722Bp)

Correlation between efflux pump genes and MIC:

Our study found significant reduction in MIC meropenem after addition of pump inhibitor as regard mexA and combined mex A and B (Table 3). There were no significant change in MIC as regard mex B alone or oprD genes.

Table 3: Correlation between efflux pump genes and MIC of meropenem

Gene	MIC 1	MIC2	P-value
mexA	>512	256	0.01
MexA+B	>512	256	0.01
mexB	256	256	>0.5
oprD	32	24	>05
Control Strain	24	24	>0.5

MIC1: without efflux pump inhibitor

MIC 2: After efflux pump inhibitor

DISCUSSION

Catheter associated urinary tract infection is considered as the commonest complication of indwelling catheter. Chronic indwelling catheters are an important reservoir of different multiresistant gram-negative organisms as *Pseudomonas aeruginosa*.²¹Our study was carried out on patient with CAUTI who were admitted to Urology Department at Tanta University Hospital. Three hundred and fifty urine samples were collected and bacteriologically studied.

We found that *Pseudomonas aeruginosa* was isolated from 14.6% of urine samples in accordance to Syed et al,²² who found *Pseudomonas aeruginosa* in 12.5% of CAUTI.

Sabir et al,²³ found *Pseudomonas aeruginosa* in only 8% of CAUTI. This may be explained by difference in infection control protocol. In our study we found that

antibiogram to *Pseudomonas aeruginosa* was as follow: All isolates were resistant to cephoperazone. Most isolates 82.5% were resistant to chloramphenicol, while 25% were resistant to Amikacin, 35% to aztreonam, 50% to gentamycin, 11% to ciprofloxacin, 8% to norfloxacin, 33% to ceftazidime and 28% to cefepime in accordance to Lister et al,^{23,24} who found resistance to amikacin ,azetronam , gentamycin and ciprofloxacin 30,42,8 and 65% respectively. In our study we found that 19.6% of *Pseudomonas aeruginosa* isolates from CAUTI were resistant to meropenem. This was in accordance to Hong et al.²⁵, who found resistance among *Pseudomonas aeruginosa* isolates in a percentage of 19.7%. Our Study disagreed with Mona et al.²⁶ who found higher percentage of resistance among *Pseudomonas aeruginosa* isolates, this may be due to the difference in the type of specimens and may be due to the difference in the underlying mechanism of resistance studied in this research. Our study disagreed with Salabi et al²⁷, who recorded high percentage of resistance to imipenem and meropenem (97.7%). This different pattern of resistance may be due to probable overuse of broad-spectrum antibiotics like carbapenems. We found that the main mechanism to meropenem resistance was via efflux pump activation and this was proved phenotypically by addition of pump inhibitor with 100% correlation between phenotypic and genotypic detection. This was in accordance to Henwood et al,²⁸ who found overexpression of mexAB-oprM efflux system in all meropenem resistant isolates of *Pseudomonas aeruginosa*.

CONCLUSION & RECOMMENDATION

Choice of detection method and treatment protocol is mandatory to control multiresistant *p-aeruginosa* with overexpression of efflux pump.

If all or any of the efflux pump genes were positive, then the dosage of antibiotic should be increased in addition with pump inhibitors should be administered according to the patient condition.

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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