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ORIGINAL ARTICLE

Detection of Efflux Pumps in Carbapenem Resistant *Pseudomonas Aeruginosa* Isolated from Benha University Hospitals

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ABSTRACT

Background: *Pseudomonas aeruginosa (P. aeruginosa) infections pose a significant risk to hospitalized patients due to its high prevalence and inherent resistance mechanisms. Efflux pumps, particularly the Mex family, contribute significantly to multidrug resistance (MDR) in P. aeruginosa.* ***Objectives:*** *To detect phenotypic and genotypic efflux pump-mediated antibiotic resistance in P. aeruginosa isolated from patients admitted to Benha University Hospitals.* ***Methodology:*** *This cross-sectional descriptive study was executed on 50 P. aeruginosa strains isolated from different clinical samples of patients admitted to hospitals of Benha University. The isolates underwent identification and antimicrobial susceptibility testing by means of VITEK® 2 compact system. The phenotypic identification of the efflux pumps was done by the Carbonyl Cyanide M-Chlorophenyl Hydrazone test (CCCP Test), followed by identification of different genes encoding efflux pump-mediated carbapenem resistance by PCR assays.* ***Results:*** *from the different clinical samples, P. aeruginosa were isolated mostly from sputum samples (30%), wound swabs (24%) and urine samples (22%). P. aeruginosa isolates exhibited varying sensitivities to tested antibiotics, showing the highest sensitivity to colistin (70%). The Minimum Inhibitory Concentration (MIC) of meropenem and imipenem significantly decreased in the existence of CCCP, indicating efflux pump involvement. PCR results revealed a high prevalence of MexA (72.4%) and MexE (79.3%) genes, correlating well with phenotypic findings.* ***Conclusion:*** *Efflux pumps have a significant function in mediating antibiotic resistance in P. aeruginosa isolates. The correlation between phenotypic and genotypic results, particularly the prevalence of MexA and MexE genes, underscores the importance of efflux pumps in MDR in P. aeruginosa strains from this clinical setting.*

INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) infections are frequent, with hospitalised patients bearing most of the infection burden. As stated by the National Nosocomial Infections Surveillance (NNIS) System, *P. aeruginosa* is the second most frequently isolated organism in nosocomial pneumonia (17 %), the third most frequently isolated organism in both surgical site infections (11 %) and urinary tract infections (UTI), and the fifth most frequently isolated organism across all nosocomial infection sites (9 %) [1].

Opportunistic pathogen *P. aeruginosa* causes illness in healthy individuals infrequently. The differential diagnosis frequently includes this organism in consideration for a variety of gram-negative illnesses. It is frequently associated with serious and sometimes fatal nosocomial infections, particularly in immunocompromised hosts. Progressive lung illness results from chronic infection in cystic fibrosis, which is sometimes aggravated through the emergence of antibiotic resistance. *Pseudomonas aeruginosa* has the potential to induce infection in a healthy host through inadequate inoculum or epithelial rupture trauma. Although animal models indicate both humoral and cell-mediated immunity have a function in the host's defence against *P. aeruginosa*, neutrophil-mediated immunity is the most crucial host defence mechanism [1].

P. aeruginosa can exhibit intrinsic, acquired, and adaptive antibiotic resistance mechanisms; occasionally, all three can be seen in a single strain. resulting in MDR *P. aeruginosa* infections, which are directly associated with increased rates of morbidity, mortality, lengthier hospital stays, and greater costs of medical care [2].

Amp C, a naturally occurring inducible β-lactamase, reduced outer membrane permeability, and constitutive production of membrane efflux (Mex) pumps are the causes of *P. aeruginosa* intrinsic resistance [3].

The genome of *P. aeruginosa* has twelve possible mex family efflux systems. Of these, four multicomponent MDR resistance nodulation division (RND) efflux pumps (mexEF-OprN, mexXY-OprM, mexCD-OprJ, and mexAB-OprM) are the best described as antibiotic transporters in this organism. Some are regarded as a primary source of MDR because

they can expel a variety of antibiotics and are constitutively expressed [4].

MDR is facilitated by the efflux pump mechanism, which eliminates a variety of compounds and medicines, as well as colours, organic solvents, detergents, molecules required for intercellular communication, and metabolic products. *P. aeruginosa* has an efflux pump transporter that is part of the resistance nodulation division family. It is made up of three components: the transporter, the linker, and the outer membrane pore. This mechanism prevents the compound that has been extruded from staying in the periplasm and returning to the cytosol [5].

The goal of this study was to phenotypically and genotypically identify P. aeruginosa isolates from patients that exhibited efflux pump-MDR.

METHODOLOGY

This cross-sectional descriptive study was executed in Medical Microbiology and Immunology Department, Benha Faculty of Medicine, Benha University during period from January 2021 to September 2022 after revision and approval **(approval code Ms 23-12-2021)** of Benha Faculty of Medicine's Ethics Committee's study protocol.

This research was executed on 50 strains of *P. aeruginosa* isolated from 115 different clinical samples collected from 115 patients (80♂ and35♀) admitted to Burn unit, Urology, Internal Medicine, Surgery Wards and Intensive Care Unit (ICU) of Benha University Hospitals.

Every participant provided a written, informed consent after complete explanation of the benefits and scope of the study. Each specimen was assigned a code number to protect participant privacy and data confidentiality.

Samples were sent as quickly as possible to the medical department's laboratory for immunology and microbiology.

All samples were cultured on MacConkey's agar and Pseudomonas Agar P plates (Indomedix, Egypt) and incubated at 37℃ for 24 hours aerobically, identification of *P. aeruginosa* was done by colonial morphology, microscopic examination of Gram-stained films, oxidase test, fluorescein production[6], growth at 42°C[7], examination of motility[8] and gelatine liquefaction test [8].

Antimicrobial susceptibility (AST) testing by VITEK® 2 compact system:

Antibiotic susceptibility was done for the isolated *P. aeruginosa* strains by using VITEK® 2 antibiotic susceptibility cards. ***(Biomerieux).*** Automated testing utilising the minimal inhibitory concentration (MIC) method as described by MacLowry, Marsh, and Gerlach is the basis of the AST card for VITEK® 2 Systems ***(VITEK® 2 Systems Product Information, 2008).***

Phenotypic detection of efflux pump by Carbonyl Cyanide M-Chlorophenyl Hydrazone test (CCCP Test):

The isolates that demonstrated antibiotic resistance to carbapenem underwent phenotypic efflux pump identification using (CCCP Test) [9].

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

Meropenem and imipenem's MICs, as determined by the agar dilution technique, varied from 0.25 to 128 mg/L. As per the CLSI-recommended breakpoint values, *P. aeruginosa* isolates having MIC values ≥ 8 mg/L are classified as meropenem-resistant. MICs were repeated in the presence of concentration of 10 µM of the efflux pump inhibitor CCCP (prepared by adding 2mg of CCCP to one liter of the agar). Antibiotic resistance may be influenced by the existence of an efflux system, as evidenced by the decrease in MIC of antibiotics containing CCCP.

Molecular detection of mexA, mexE and mexX genes[10]:

DNA was extracted from bacteria and its concentration was measured then the primers specific to each gene (table 1) was prepared and DNA amplification by Conventional PCR was done.

Table 1: Primer sequence of mexA, mexE and mexX genes

Gene	Primers	Base Pair
mexA	F:GGCGACAACGCGGCGAAGG R: CCTTCTGCTTGACGCCTTCCTGC	332
mexE	F:TCATCCCACTTCTCCTGGCGCTACC R:CGTCCCACTCGTTCAGCGGTTGTTCGATG	260
mexX	F:AATCGAGGGACACCCATGCACATCC R:CCCAGCAGGAATAGGGCGACCAG	114

Statistical analysis

The Kolmogorov-Smirnov test was utilized as a means of assessing normality. Quantitative data were expressed using mean ± standard deviation (SD) while categorical data were presented as frequency and percentage. Statistical comparisons were conducted utilizing Mann-Whitney U-test. Wilcoxon-signed rank test was utilized to determine the relationship between related non-parametric quantitative variables. Agreement was tested using the Kappa (κ) agreement coefficient.

RESULTS

This research was executed on 50 *P. aeruginosa* strains isolated from 115 specimens collected from 115 patients (80♂ and35♀) admitted to Burn unit, Urology, Internal Medicine, Surgery Wards, Intensive Care Unit (ICU) of Benha University Hospitals, Benha Faculty of Medicine.

Fifty strains of *P. aeruginosa* were isolated from different samples in the following order: sputum samples (30%), wound swab (24%), urine (22%), ear swab (14%), Catheter tip (6%) and at last pus (4%). Figure (1)

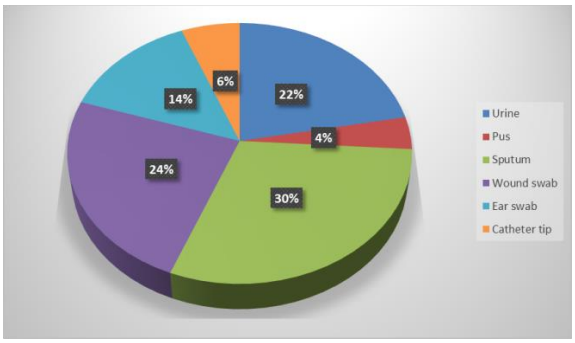

Fig. 1: Percentage of isolated *P. aeruginosa* from Clinical samples obtained in the current study

The 50 *P. aeruginosa* strains were tested for antibiotic susceptibility utilizing the VITEK® 2 compact system, colistin showed the highest sensitivity in 70% of the tested isolated *P. aeruginosa* strains, followed by aztreonam in 46%, meropenem in 44%, imipenem in 42%. The least sensitivity was reported with gentamycin (8%). Figure (2)

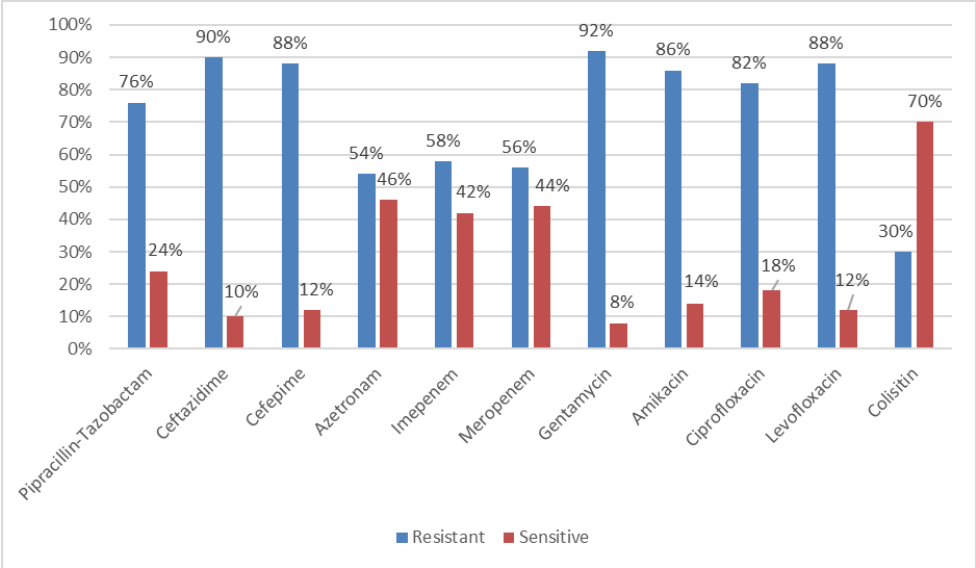

Fig. 2: Antimicrobial susceptibility testing for isolated P. aeruginosa strains by VITEK® 2 compact system

The mean MIC with meropenem without CCCP was 20.621 ± 21.311 mg/l (range 0.5-64 mg/l) that was statistically significantly higher as compared to the MIC for meropenem with CCCP (6.328 ± 6.0934 mg/l) (range 0.5-24 mg/l). The mean MIC for imipenem without CCCP was 13.431 ± 14.783 mg/l (range 0.25-64 mg/l) that was statistically significantly higher as compared to the MIC with imipenem with CCCP (6.50 ± 7.426 mg/l) (range 0.25-32 mg/l). The result is shown in (Table 2)

Table 2: Comparison between the Minimum inhibitory concentration (MIC) of isolated *P. aeruginosa* strains in Meropenem and Imipenem with and without CCCP.

Meropenem	MIC in absence of CCCP (mg/L)	MIC in presence of CCCP (mg/L)	Test of significance
Mean ± SD	20.621 ± 21.311	6.328 ± 6.0934	z *= - 4.021
Median (Range)	16 (0.5 – 64)	4 (0.5 – 24)	**P < 0.001 * (HS)**
Imipenem			
Mean ± SD	13.431 ± 14.783	6.50 ± 7.426	z* = - 4.028
Range	8 (0.25 – 64)	4 (0.25 – 32)	**P < 0.001* (HS)**

Continuous data stated as mean±SD and median (range), *z: Wilcoxon signed rank test, *HS: Highly significant (p≤ 0.001).

The mean MIC for meropenem without CCCP was 20.621 ± 21.311 mg/l (range 0.5-64 mg/l) that was statistically significantly higher as compared to the MIC with Imipenem without CCCP (13.431 ± 14.783 mg/l) (range 0.25-64 mg/l). The mean MIC for meropenem with CCCP was 6.328 ± 6.093 mg/l (range 0.5-24 mg/l) with no statistically significant difference as compared to the MIC with Imipenem with CCCP (6.50 ± 7.426 mg/l) (range 0.25-32 mg/l). (Table 3)

Table 3: Comparison between the Minimum inhibitory concentration for *P. aeruginosa* strains in Meropenem and Imipenem without CCCP and with CCCP.

without CCCP	MIC of Meropenem without CCCP (mg/L)	MIC of Imipenem without CCCP (mg/L)	Test of significance
Mean ± SD	20.621 ± 21.311	13.431 ± 14.783	z*= - 2.073
Median (Range)	16 (0.5 – 64)	8 (0.25 – 64)	P = 0.038 * (S)
with CCCP			
Mean ± SD	6.328 ± 6.093	6.50 ± 7.426	*z = - 0.331
Range	4 (0.5 – 24)	4 (0.25 – 32)	P = 0.741 (NS)

*z: Mann-Whitney U test, *S: Significant (p≤ 0.05)

Regarding the identification of genes encoding efflux pump mediated carbapenem resistance by PCR assay for 29 MDR *P. aeruginosa* strains, Mex A was positive in 72.4%, Mex E was positive in 79.3% and Mex X was positive in 48.3%. (Table 4), Figure (3)

Table 4: Detection of genes encoding efflux pump mediated carbapenem resistance by PCR:

Gene type	PCR results	Number of tested isolated P. aeruginosa strains N= 29	Percentage (%)
Mex A gene	**Positive**	21	72.4%
	Negative	8	27.6%
Mex E gene	**Positive**	23	79.3%
	Negative	6	20.7%
Mex X gene	**Positive**	14	48.3%
	Negative	15	51.7%

Categorical data expressed as Number (%)

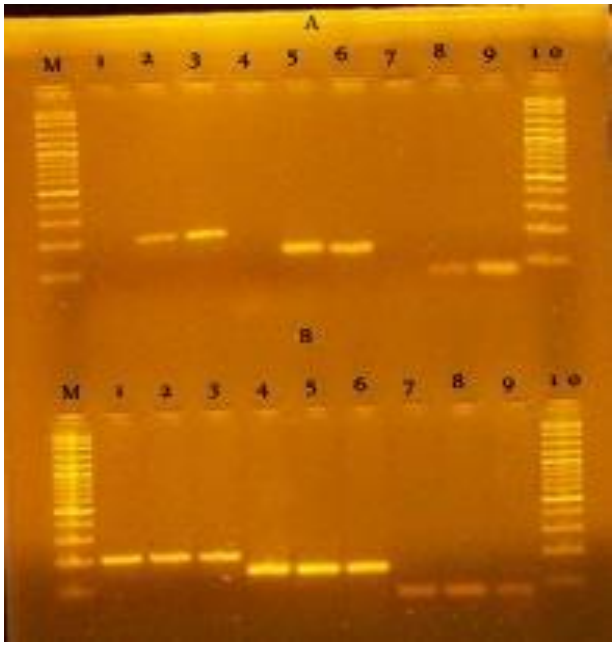

Fig. 3: Detection of genes encoding efflux pump mediated carbapenem resistance by PCR. (L anes M& 10) ladder (3000Bp) (A) Positive Lanes 2 &3 for mexA gene (332Bp), positive lanes 5&6 for mexE gene (260Bp), positive lanes 8&9 for mexX gene (114Bp). (B) Positive Lanes 1,2 &3 for mexA gene (332Bp), positive lanes 4,5&6 for mexE gene (260Bp), positive lanes 7,8&9 for mexX gene (114Bp)

There was a strong concurrence between the phenotypic results and genotypic outcomes (Mex A) (κ= 0.840, P < 0.001). Also, there was a moderate concurrence between the phenotypic results and genotypic outcomes (Mex E) (κ= 0.663, P < 0.001). And finally, there was a mild concurrence between the phenotypic results and genotypic outcomes (Mex X) (κ= 0.386, P =0.027). as shown in Table 5.

Table 5: Agreement between phenotypic results and genotypic results of *P. aeruginosa* for (Mex A), (Mex E) and (Mex X).

Mex A	Phenotypic results N = 29		Genotypic results (Mex A) N = 29		Test of significance
	Number	Percent	Number	Percent	
Negative	10	34.5 %	8	27.6 %	***κ= 0.840**
Positive	19	65.5 %	21	72.4 %	***P < 0.001***
Mex E					
Negative	10	34.5 %	6	20.7 %	***κ= 0.663**
Positive	19	65.5 %	23	79.3 %	***P < 0.001***
Mex X					
Negative	10	34.5 %	15	51.7 %	***κ= 0.386**
Positive	19	65.5 %	14	48.3 %	***P = 0.027***

Categorical data expressed as Number (%), *κ: Kappa agreement coefficient, *: Statistically significant

DISCUSSION

In our study, *P. aeruginosa* was isolated mostly from sputum samples (30%), wound swabs (24%), urine (22%), ear swab (14%), catheter tip (6%), and pus (4%) of the obtained specimens.

In similar Egyptian research performed by Wassef et al.[11] lower respiratory tract infections were the primary reason for *P. aeruginosa* isolations (44.2 %), then came surgical site infections (37.5 %) and UTI (23 %). The research of Abdallah et al.[12] reported that 44%, 36%, 18% and 2% of the specimens were collected from endotracheal aspirate, urine, pus, and blood, respectively. Also, Mohamed and Abdelhamid[13] revealed that the primary cause of the 33.3% isolation rate was endotracheal aspirate (29%), urine (27%), burn (25%), and other sources (19 %).

Variable findings were revealed by Elbrolosy et al.[14] who discovered that the highest isolation rate of *P. aeruginosa* was from burn swabs (57.5%), blood cultures (17.5%) and sputum samples (8.3%) followed by bronchial aspirates, urine, wound swabs and finally ascetic fluid samples.

Antibiotics-resistant *P. aeruginosa* isolates from nosocomial infections increase, it is important to regularly study the isolates' resistance to widely used antibiotics to make the best selection of antimicrobials used for the treatment of these illnesses[15].

Concerning the antimicrobial susceptibility testing in our study, our findings aligned with other findings, Elbrolosy et al.[14] discovered that a significant proportion of the acquired *P. aeruginosa* isolates (73.3%) were resistant to both imipenem and meropenem. Also, an Egyptian study that was conducted by Abaza et al.[16] described that 78.3% of isolates were resistant to imipenem and 73.7% were resistant to meropenem. Likewise, Diab et al.[17] found 72% of *P. aeruginosa* isolates had imipenem resistance while Taher et al.[18] in a study from Iran, also reported high resistance rates to imipenem; and highest antibacterial activity for colistin.

In Saudi Arabia, 38.57% of *P. aeruginosa* isolates were discovered to be imipenem-resistant in the investigation conducted by Mohamed et al.[19]. This could be due to the altered bacterial behaviour across time.

When CCCP is used as an efflux pump inhibitor, the MIC is significantly reduced upon addition. CCCP demonstrates how efflux pumps contribute to imipenem/meropenem resistance[20]. Therefore, the isolates that showed resistance to meropenem or imipenem by VITEK method in our study were exposed to a phenotypic efflux pump identification procedure, which involved adding CCCP to Mueller-Hinton agar and then estimating MIC. The mean MIC of meropenem/ imipenem negative efflux pump phenotype was significantly increased as compared to the MIC with positive efflux pump phenotype ($p<0.001$). This was consistent with Kishk et al.[21] and Khalek et al.[22] who discovered that the primary mechanism underlying carbapenem resistance was efflux pump activation, which was phenotypically validated by the addition of CCCP as a pump inhibitor. This also resembled the outcomes of Choudhury et al.[23] who had observed phenotypic efflux pump activity and a significant drop in MIC in all of their isolates of *P. aeruginosa* upon addition of CCCP.

Concerning the mean MIC of meropenem without and with CCCP, *P. aeruginosa* contains genes that encode the MexCD-OprJ, MexEF-OprN, multidrug efflux pumps (Mex) MexXY, and MexAB-OprM[24].

As regard PCR result in our study in order to identify genes encoding efflux pump mediated carbapenem resistance (Mex A, Mex E, and Mex X), several previous studies have assessed the genotypes of carbapenem-resistant *P. Aeruginosa*, with Mex A gene the most predominantly studied. Abdallah et al.[12] found that Mex A gene overexpression was detected in 54.2% of isolates. Elbrolosy et al.[14] reported the overexpression of Mex A and Mex X genes in 77.6% and 61% of the isolates, respectively.

Overexpression of Mex A gene was observed in 55.5% and 51% of the isolates in the studies of Pourakbari et al.[25] and Murugan et al.[26], respectively. In addition, Tomas et al.[27] determined that a rise in Mex A expression was the primary meropenem resistance mechanism.

The present study demonstrated that the agreement between the phenotypic and genotypic results was strong in Mex A-overexpressed isolates ($p<0.001$), moderate in Mex E-overexpressed isolates ($p<0.001$), and mild with the Mex X-overexpressed isolates ($p=0.027$).

The findings of the present study concerning the different levels of concurrence between phenotypic and genotypic outcomes of carbapenem resistance in *P. aeruginosa* isolates based on the expression of MexA, MexE, and MexX efflux pump systems, can be explained by considering that other factors beyond efflux pump expression can influence carbapenem resistance. For instance, mutations in other genes, like those involved in outer membrane permeability or carbapenemase production, can impact resistance levels and contribute to variations in agreement [28].

Also, *P. aeruginosa* infected populations can exhibit genetic heterogeneity. This means that not all isolates within a population might over express the same efflux pump systems to the same degree, leading to differences in resistance patterns. Moreover, differences in testing methods and conditions could lead to variations in observed resistance. Finally, the clinical context should not be ignored since the isolates used in this study was obtained from different clinical sources and patient populations. Variations in clinical context, such as patient demographics, comorbidities, and prior antibiotic exposure, can influence resistance mechanisms and expression levels.

CONCLUSION

Efflux pumps have a significant function in mediating antibiotic resistance in *P. aeruginosa* isolates. The correlation between phenotypic and genotypic results, particularly the prevalence of MexA and MexE genes, underscores the importance of efflux pumps in multidrug resistance in *P. aeruginosa* strains from this clinical setting.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
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