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RESEARCH PAPER

Phenotypic and Genotypic Detection of Efflux Pump in Meropenem-Resistant *Pseudomonas aeruginosa* Among Burn Patients

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Abstract

Background: Multidrug-resistant (MDR) *Pseudomonas aeruginosa* in burn wounds is becoming a difficult issue worldwide. The recent emergence of meropenem-resistant MDR *Pseudomonas aeruginosa* poses a serious threat to public health. The activity of the MDR efflux pump is a key mechanism contributing to meropenem resistance.

Objective: The study aims to detect efflux pump phenotypically and genotypically in meropenem-resistant MDR *Pseudomonas aeruginosa* among burn wound patients.

Methods: A total of 120 laboratory isolates of *Pseudomonas* species from burn wounds were collected from the patients of National Institute of Burn and Plastic Surgery, Dhaka. The automated VITEK® 2 Compact system in the department of microbiology and immunology at Bangladesh Medical University (BMU) was used to identify *Pseudomonas aeruginosa*. Antimicrobial susceptibility testing and identification of MDR *Pseudomonas aeruginosa* were conducted by using the Kirby-Bauer disk diffusion technique. Out of 120 laboratory isolates of *Pseudomonas* species; *Pseudomonas aeruginosa* accounted for the majority of these isolates (109, 90.8%), followed by *Pseudomonas fluorescens* (6, 5%) and *Pseudomonas putida* (5, 4.17%). Among 109 *Pseudomonas aeruginosa,* 92 (84.4%) were multidrug resistant (MDR). Among 92 MDR cases, 78 (84.8%) were meropenem-resistant *Pseudomonas aeruginosa*. Finally, 78 meropenem-resistant *Pseudomonas aeruginosa* among all MDR were selected as study subjects. Efflux pump that causes meropenem resistance was identified both phenotypically and genotypically. Ethidium bromide cartwheel (EtBr-CW) approach was used for phenotypic identification and efflux pump genes (MexAB-OprM) detection by conventional polymerase chain reaction (PCR) was used for genotypic identification of efflux pump.

Result: Out of 109 *Pseudomonas aeruginosa* isoates, majority (92, 84.4%) of *Pseudomonas aeruginosa* were multidrug-resistant (MDR) where majority (78, 84.8%) showed resistance to meropenem. Among meropenem-resistant *Pseudomonas aeruginosa* (78); efflux pump was detected both genotypically and phenotypically in 52 (66.7%) cases and only genotypically in 05 (6.4%) cases. Therefore, in 57 (73.1%) cases efflux pump was detected genotypically through detection of efflux pump gene. Among the different efflux pump gene; Mex A gene was found in 52 cases, Mex B gene in 57 cases, Opr M gene in 19 cases, both Mex A and Mex B gene in 51 cases and Mex A, Mex B, Opr M gene together in 19 cases.

Conclusion: The majority of pseudomonas aeruginosa were multidrug resistant (MDR) and showed resistant to meropenem. Efflux pump genes (MexA, MexB, OprM) were found in good number of the patients.

Keywords: Efflux pump, Meropenem, Multidrug resistant *P. aeruginosa.*

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Introduction

Pseudomonas aeruginosa is a major contributor to healthcare-associated infections, particularly in hospitalized patients with burn injuries.[1] Due to its ability to flourish on moist burn wound surfaces and

endure in hospital settings, it has been identified as a prominent colonizer of burn wounds.[2] It is considered the third nosocomial pathogen [3] responsible for 10–15% of nosocomial infections.[4]

A breach in the protective skin barrier, reduced immunity, and prolonged hospital stay are important factors responsible for infection of burn wounds with such opportunistic pathogens, especially multi-drug resistant (MDR) *Pseudomonas aeruginosa.*[1] These MDR bacterial isolates can cause dangerous complications like bacteremia, septicemia, and even death in cases of burn infection[5]. Patients with septicemia had mortality rates of up to 37% in burn wound patients infected with MDR *Pseudomonas aeruginosa* (*P. aeruginosa)* infection.[6]

Centers for Disease Control and Prevention (CDC) demonstrated MDR *P. aeruginosa* as a "serious" pathogen.[7] In Bangladesh, the average rate of MDR organisms increased gradually by two-fold between 2016 and 2018, and became 62% in 2019 & in the case of *P. aeruginosa,* it is 83.4%.[8] It had been shown that the rate of MDR *P. aeruginosa* in wound-infected patients is 72.5%, 76.8% and 33.3% in Bangladesh, India, and Africa, respectively. [9-11]

Across the globe, there has been a significant increase in both the use of carbapenems and the emergence of *P. aeruginosa* that is resistant to them. The World Health Organization (WHO) designated carbapenem-resistant *P. aeruginosa* (CRPA) as "high-risk pathogens".[12] A significant increase in resistance of carbapenems to *P. aeruginosa* ranges from 30.1 to 74% worldwide.[13] Carbapenem-resistant MDR *P. aeruginosa* are 64% and 61% in Bangladesh and India.[9,10]

Antimicrobial resistance in *P. aeruginosa* is linked to three basic types of mechanisms: intrinsic, acquired, and adaptive resistance. The intrinsic antimicrobial resistance of *P. aeruginosa* is a result of several factors, including the expression of efflux pumps, restricted outer membrane permeability, and the emergence of enzymes that inactivate antibiotics. One major resistance mechanism involves efflux pumps—specialized transporter proteins that expel antibiotics from the bacterial cell.[13] This reduces the drug concentration inside the cell and allows bacteria to adapt and develop resistance through mutations or other changes. [14]

Efflux pump activity can be evaluated using both phenotypic and genotypic approaches. Phenotypic methods assess the functional activity of efflux pumps, while genotypic techniques enable detection of the presence or absence of efflux pump–encoding genes and can also determine their expression levels. Although genotypic methods are considered more specific and confirmatory, they are relatively time-consuming and require specialized laboratory facilities. Therefore, phenotypic assays often serve as a practical alternative, particularly in routine diagnostic settings.

Efflux pumps can be detected phenotypically using the Ethidium Bromide–Agar Cartwheel method or by employing efflux pump inhibitors (EPIs). The Ethidium Bromide Cartwheel assay (EtBr-CW) is a simple, cost-effective, and instrument-free method that allows simultaneous screening of up to twelve bacterial isolates with minor procedural modifications.[18]

There are various types of efflux pumps genes. In *P. aeruginosa*, the most common method is extrusion of drugs through the multidrug efflux system of the RND superfamily. Many RND superfamilies' operons, including MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexGHI-OpmD, and MexVW-OprM, are included in *P. aeruginosa's* genetic makeup and are in charge of both intrinsic and acquired multidrug resistance.[15] Because MexAB-OprM was the first RND pump to be identified that could target several types of antibiotics as well as common wild-type strains, it has multidrug resistance. Among Carbapenems, only meropenem is the substrate of MexAB-OprM.[16] In India, Efflux pump-mediated MDR *P. aeruginosa* was found in about 34.8%.[17]

Burn units harbor multidrug-resistant strains of *P. aeruginosa,* which can colonize burn wounds and lead to infection. It has become a major problem in the control of infections, particularly in developing countries.[19] The increasing frequency of carbapenem-resistant *P. aeruginosa* (CRPA) has recently become a worldwide serious /concern.[20] The upregulation of multidrug efflux pumps is one of the main causes of it.[21] To the best of our knowledge, adequate information is not available about efflux pump-mediated meropenem resistance in MDR *P. aeruginosa* in burn wounds in our country.

Materials and Methods

This cross-sectional study was conducted from March 2024 to February 2025. Our study was on 78 meropenem resistant *Pseudomonas aeruginosa*. For this, we had taken total 120 *Pseudomonas* spp.

isolates obtained from overnight cultures of burn wound swab samples with a colony count ≥ 10^4 CFU [22] at the microbiology laboratory, National Institute of Burn and Plastic Surgery (NIBPS), Dhaka, Bangladesh. Confirmatory identification of *Pseudomonas* species was performed using the VITEK® 2 Compact automated system with a gram-negative card in the department of microbiology and immunology, Bangladesh Medical University (BMU). Out of one hundred and twenty *Pseudomonas* species isolated from burn wounds; *Pseudomonas aeruginosa* accounted for the majority of these isolates (109, 90.83%), followed by *Pseudomonas fluorescens* (6, 5%) and *Pseudomonas putida* (5, 4.17%). Among 109 *Pseudomonas aeruginosa,* 92 (84.4%) were multidrug resistant (MDR). Among 92 MDR cases, 78 (84.8%) were meropenem-resistant *Pseudomonas aeruginosa*.

The susceptibility of *Pseudomonas aeruginosa* was assessed by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (HI-Media, India). Antibiotic discs were obtained from Bio Maxima, Poland, and quality control was ensured using *Pseudomonas aeruginosa* ATCC® 27853. Interpretations were based on CLSI (2024) guidelines. MDR was defined as acquired non-susceptibility to at least one agent in three or more classes of antimicrobials.

Phenotypic detection of Efflux pump activity was determined by the Ethidium Bromide Cartwheel (EtBr-CW) method.[18] Trypticase Soy Agar plates containing 1.5 mg/L ethidium bromide were divided into six sectors, inoculated with standardized bacterial suspensions in a cartwheel pattern, and incubated at 37 °C for 16 hours. Plates were examined under a UV trans-illuminator. *Pseudomonas aeruginosa* ATCC® 27853 served as the negative control.

Genotypic detection of MexAB-OprM Efflux Pump Genes was done by conventional PCR. DNA was extracted using by boiling method. Two colonies from MacConkey agar were transferred into 500 µL distilled water at 100 °C for 10 minutes, then centrifuged at 10,000 rpm for 5 minutes. A 250 µL aliquot of the supernatant was stored at –20 °C.[23] Each 20 µL PCR reaction contained 15 µL master mix, 0.15 µL Taq polymerase, 1 µL of each primer, and 5 µL DNA template. PCR products were resolved on a 1.5% agarose gel prepared in 1× TAE buffer with 6 µL of 1% ethidium bromide per 100 mL. Ten µL samples and a 100 bp DNA ladder (Geneon, Germany) were loaded and run at 120 V for 48 minutes (Biometra, Germany). DNA bands were visualized under UV light and compared to the ladder for size determination. Primer and conditions were listed below in tables (I) and (II)

All collected data presented as numbers and percentages. All statistical analyses were conducted using the Statistical Package for Social Science (SPSS), version 27 (IBM Corp., Armonk, New York).

Results

Among the 120 laboratory isolates of *Pseudomonas* species*;* the *Pseudomonas aeruginosa* accounted for the majority of these isolates (109, 90.8%), followed by *Pseudomonas fluorescens* (6,5%) and *Pseudomonas putida* (5, 4.17%). Among 109

Table I: Primer sequence and amplification size used in PCR for MexAB-OprM

Genes	Initial denaturation	Denaturation	Annealing	Extension	Cycle	Final extension
MexA	94ºC 5 min	94ºC 30 sec	55ºC 30sec	72ºC 1.5min	35	72ºC 7min
MexB	95ºC 5 min	95ºC 30sec	54ºC 30sec	72ºC 1 min	30	72ºC 7min
OprM	94ºC 5 min	94ºC 30sec	55ºC 30sec	72ºC 1.5min	35	72ºC 7min

Table II: PCR run protocol for meropenem resistant MexAB-OprM genes

Genes	Sequences		Size (bp)
Mex A	F: CTACGAGGCCGACTACCAGA	R: TGCAGGCCTTCGGTAATGAT	722
Mex B	F: ACTTCTTCAGCTTCAAGGAC	R: GAGCATGAGGAACTTGTTG	155
Opr M	F -TACCAGAAGAGTTTCGACCTGAC	R -CATGTGTCAAAACAGTCACCTCC	812

Pseudomonas aeruginosa, 92 (84.4%) were multidrug resistant (MDR). Among 92 MDR cases, 78 (84.8%) were meropenem-resistant *Pseudomonas aeruginosa* (table-III & IV).

The MDR were found in 92 (84.4%) among 109 *Pseudomonas aeruginosa*, in which 78 (84.8%) showed meropenem resistance and 14 (15.2%) isolates showed meropenem sensitive (figure-1).

Table-III: Distribution of MDR including meropenem among the pseudomonas aeruginosa

Psrudomonas	P. aeruginosa	MDR P. aeruginosa	Meropenem resistant
120	109	92	78

Table IV: Antimicrobial resistance pattern of isolated *Pseudomonas aeruginosa* (N=109)

Name of antimicrobial agent	Resistance percentage
Ciprofloxacin	95(87.1%)
Gentamicin	87(79.8%)
Ceftazidim	93(85.3%)
Amikacin	81(74.3%)
Aztreonam	83(76.1%)
Meropenem	78(71.5%)
Netilimicin	71(65.1%)
Cefepime	94(86.2%)
Pipercilin-Tazobactum	79(72.4%)
Ceftazidim-Avibactum	80(73.3%)

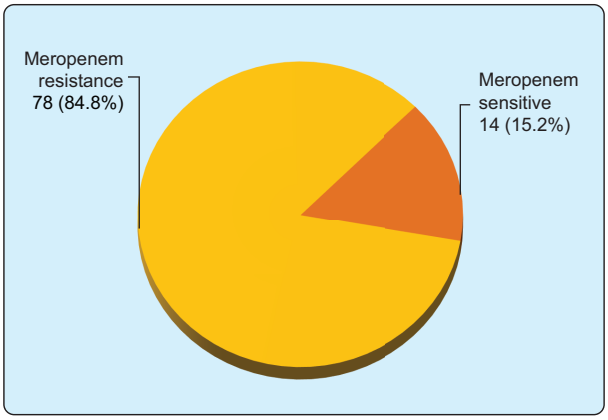


Figure 1: Distribution of Meropenem resistance among MDR *Pseudomonas aeruginosa* (N=92)

All 78 isolates were tested for efflux pump detection phenotypically using the Ethidium Bromide (Et-Br) cartwheel method. The results that 52 (66.7%) isolates give positive results that means they did not fluorescence at 1.5 mg/L concentration of Et-Br (figure-2).

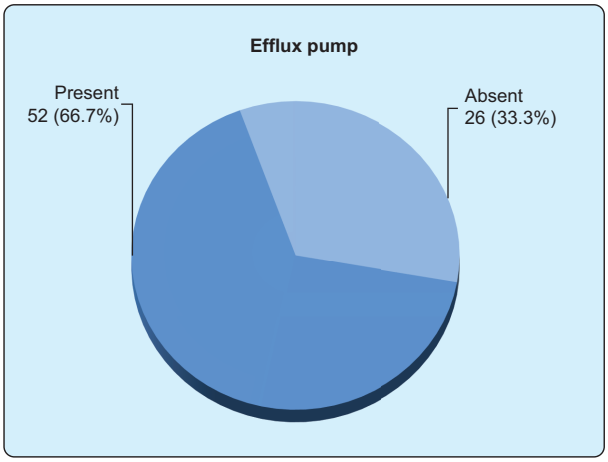


Figure 2: Distribution of phenotypically detected efflux pump activity among Meropenem resistant MDR *Pseudomonas aeruginosa*(N=78)

Table V: Frequency of efflux pump genes (genotypically detected efflux pump activity) among meropenem resistant MDR *Pseudomonas aeruginosa*(N=78)

Gene	No. of isolate positive	
	No	%
Efflux pump gene positive	57	73.1%
Mex A	52	66.6%
Mex B	57	73.1%
Opr M	19	24.0%
Mex A and Mex B	51	66.6%
Mex A, Mex B and Opr M	19	24.0%
Efflux pump gene negative	21	26.9%

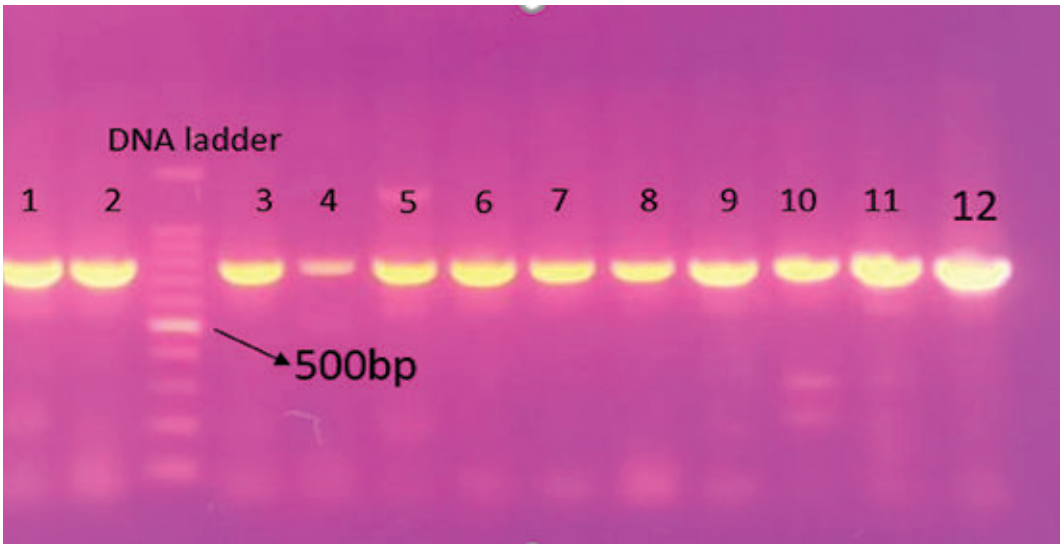
Among 78 Meropenem resistant *Pseudomonas aeruginosa*, MexAB-OprM genes found in 57 (73.1%) samples. Among them MexA gene found in 52 (66.6%) isolates, MexB gene founds in 57 (73.1%) isolates and OprM gene found in 19 (24%) isolates. Both MexA and MexB genes found in 52 (66.6%) samples and finally all three (MexA, MexB, OprM) genes found in 19 (24%) samples (table-V).

Out of 78 Meropenem resistant MDR *Pseudomonas aeruginosa,* 52 cases showed efflux pump both phenotypically and genotypically; 05 cases showed efflux pump only genotypically. Therefore, efflux pump was detected genotypically in 57 cases (table-VI).

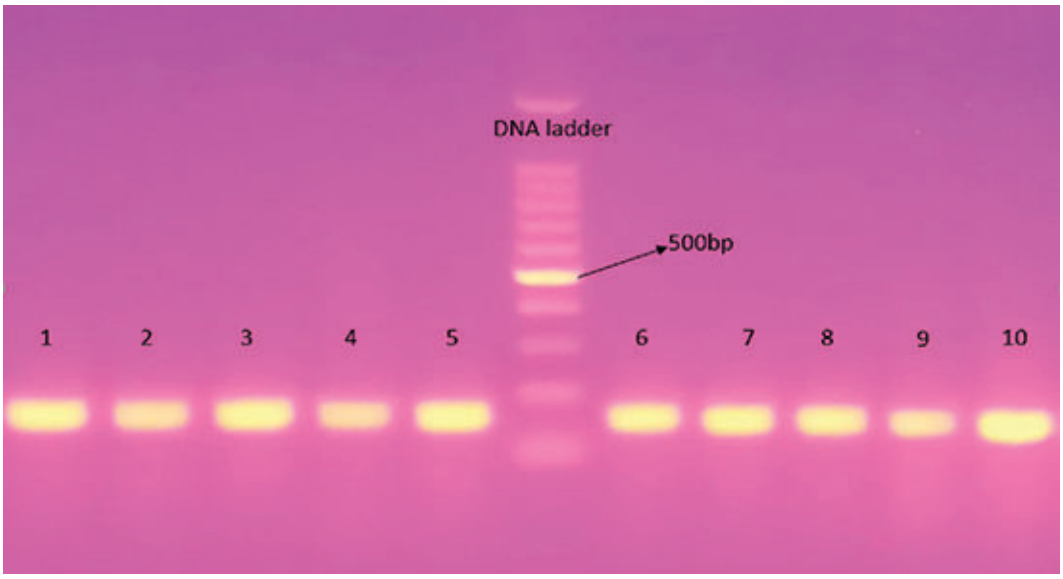
Table VI: Comparison between phenotypic and genotypic detection of efflux pump in Meropenem resistant MDR *Pseudomonas aeruginosa* (N=78)

EtBr-CW method (Phenotypic detection)	PCR for efflux pump gene (genotypic detection)		Total	
	Positive	Negative		p value
Positive	52 (66.7%)	0 (0.0%)	52	<0.001
Negative	05 (6.4%)	21 (26.9%)	26	
Total	57	21		

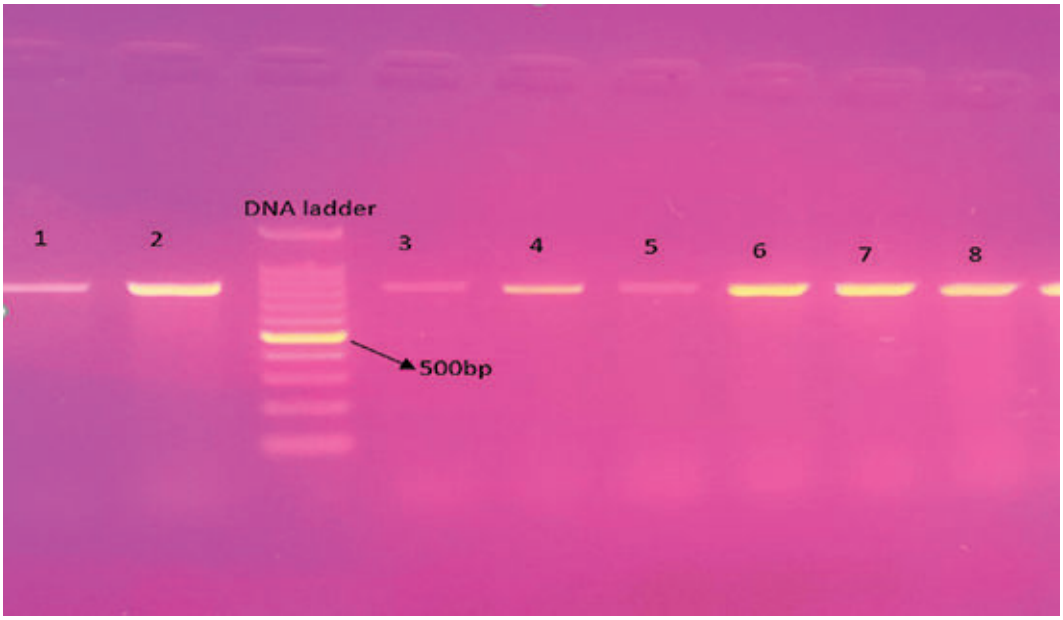
P value measured by Fisher's exact test, *P value <0.001 is significant



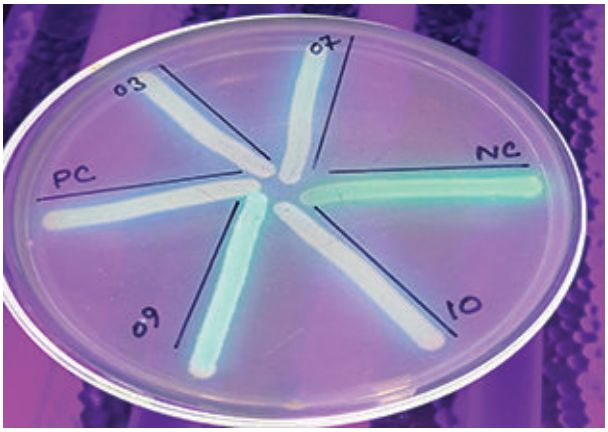
Photograph 1: Agarose gel electrophoresis analysis showed amplified DNA product of MexA (722 bp)



Photograph 2: Agarose gel electrophoresis analysis showed amplified DNA product of MexB (155 bp)



Photograph 3: Agarose gel electrophoresis analysis showed amplified DNA Product of OprM (812 bp)



Photograph 4: Isolate 03, 07, 10 shows no fluorescence 1.5mg/L ethidium bromide indicating that these isolates contain efflux pumps. *Pseudomonas aeruginosa* ATCC 27853 was used as negative control (NC)/

Discussion

Pseudomonas aeruginosa (P. aeruginosa) is a leading opportunistic pathogen responsible for diverse infections, particularly in immunocompromised hosts such as burn patients.[21] Burn units are highly susceptible to outbreaks due to prolonged hospitalization, extensive wound surfaces, and the organism's remarkable adaptability to hospital environments.[11] Its intrinsic and acquired resistance mechanisms, including efflux pump overexpression, contribute to multidrug resistance.[24]

This study investigated efflux pump–mediated resistance in meropenem-resistant *P. aeruginosa*, and identified MaxAB-OprM efflux pump genes.

Out of 120 *Pseudomonas* isolates, *P. aeruginosa* was most prevalent (90.8%), followed by *P. fluorescens* (5%) and *P. putida* (4.17%). In our study, 92 (84.4%) isolates were multidrug resistant (MDR) among 109 *P. aeruginosa*. This is similar to previous study in Bangladesh and China done by Safain *et al* (2020) and Dou *et al* (2017) respectively who showed that, 83.4% and 89.8% were MDR.[8-25] Among total 92 isolated MDR, 78 (84.7%) *P. aeruginosa* exhibited meropenem resistance in this study. This is consistent with study by Dou *et al* (2017) in China revealed 87.3% isolates exhibited meropenem resistance among MDR *P. aeruginosa* in burn patients.[25] In India, prevalence to meropenem resistance among burn patients were 74% [17] which is nearly similar to this study.

In current study, 66.7% meropenem resistant MDR *P. aeruginosa* were positive for efflux pump phenotypically by EtBr-CW method. In Iran and Egypt, prevalence of efflux pump mediated meropenem resistance in MDR *P. aeruginosa* were 62% and 65.5% respectively [14,26] which are concomitance to our current study. Genotypically, MexAB-oprM genes responsible for meropenem resistance were detected in 73.4% MDR *P. aeruginosa* in current study which is similar to china where it were 76.9% [27]. This similarity may be due to same genetic strain of *P.*

aeruginosa infection. In India lower expression of MexAB-oprM genes were showed and it was 45%.[18]

In the present study, among the MexAB-OprM efflux pump genes, MexA was detected in 91.2% and MexB in 100% of isolates, while OprM was found in 45.6% of isolates. These findings are consistent with those of Okafor & Nwodo (2023), who reported MexA in 96.5% and MexB in 100% of isolates, and with an Indian study that found OprM expression in 40% of isolates. Such similarity across different geographical regions highlights the conserved nature of MexAB-OprM genes in *P. aeruginosa*.

The current study also revealed a significant association ($p = 0.015$) between MexAB-OprM gene presence and resistance to meropenem. This aligns with an Iranian study where the association was also significant ($p = 0.0114$), reinforcing the role of MexAB-OprM in mediating meropenem resistance.

Furthermore, phenotypic analysis identified efflux pump–mediated meropenem resistance in 52 (66.7%) isolates, whereas genotypic detection of MexAB-OprM genes indicated resistance in 57 (73.1%) isolates out of 78. Notably, there was strong agreement between the two methods: 91.2% of isolates harboring efflux pump genes exhibited phenotypic overexpression, while 8.8% carried the genes but did not show phenotypic resistance. The strong correlation ($p < 0.001$) between phenotypic and genotypic methods underscores the reliability of molecular detection in confirming efflux pump–mediated resistance.

Conclusion

The majority of *Pseudomonas aeruginosa* were multidrug resistant (MDR) where again most organism showed resistance to meropenem. Among meropenem resistant organism; efflux pump mechanisms, particularly involving the *MexA*, *MexB*, and *OprM* genes were found in good number of the patients. *Pseudomonas aeruginosa* showed resistance to meropenem which is a growing threat in clinical practice. So, the importance of routine molecular screening and targeted therapeutic strategies to better manage infections caused by *Pseudomonas aeruginosa* is of paramount importance in burn patients. Advances in understanding efflux pump mechanisms have facilitated the development of efflux pump inhibitors (EPIs), which can block pump activity and restore antibiotic efficacy.

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