Research article

Phenotypic and genotypic detection of efflux-mediated carbapenem resistance in *Pseudomonas aeruginosa* clinical isolates

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Abstract

Introduction: *Pseudomonas aeruginosa* is the leading pathogen causing healthcare-associated infections and exhibits high levels of intrinsic resistance, with only a limited number of antibiotics available against it. Against this background, the present study was conducted to identify carbapenem resistance in *P. aeruginosa* mediated by efflux mechanisms and to assess the *in vitro* detection of efflux pump activity using efflux pump inhibitors as a diagnostic method.

Methods: Fifty non-duplicate multidrug-resistant *P. aeruginosa* isolates from different clinical samples were tested for meropenem (10 µg) susceptibility. All meropenem-resistant strains were tested for efflux-mediated resistance using the Carbonyl Cyanide 3-Chloro Phenylhydrazone (CCCP) phenotypic test and *mexE* gene detection by conventional PCR.

Results: All 50 *P. aeruginosa* isolates were resistant to carbapenem. Of the 12 (24%, 12/50) isolates that exhibited phenotypic resistance by the CCCP method, only seven (14%, 7/50) were *mexE* gene-positive by conventional PCR.

Conclusions: These above findings which contribute to multidrug resistance in the local setting, although they are not universally present among all MDR strains, highlight the therapeutic challenge posed by *P. aeruginosa* and the need for ongoing surveillance and institution-specific antibiotic stewardship.

Key words: *Efflux pump inhibitor, multidrug resistance, Pseudomonas aeruginosa.*

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Introduction

A primary cause of treatment failure in bacterial infections is the emergence of antibiotic resistance, which is a global health concern. Antibiotic resistance is caused by a variety of mechanisms, including altered targets, drug inactivation, reduced permeability, and enhanced efflux.[1] Efflux can make antibiotics ineffective at infection sites where inadequate drug concentration occurs, which could have a major impact in clinical settings, even though it frequently leads to a low level of resistance. Additionally, cross-resistance to different drug classes caused by Gram-negative bacteria poses challenges in identification by standard drug susceptibility testing.[2,3]

Detection of efflux-mediated resistance by phenotypic assays is frequently indeterminate due to (i) the common coexistence of other mechanisms that affect the same drug partially or completely, (ii) differences in expression of resistance in various strains, and (iii) challenges in the detection of mild to moderate resistance. Western blot or northern blot analysis has long been used for the specific detection of efflux pumps, but these techniques are difficult to apply in clinical laboratory settings.[2]

P. aeruginosa is the leading pathogen causing healthcare-associated infections and exhibits high levels of intrinsic resistance, with only a limited number of antibiotics available against it. Furthermore, it can acquire additional resistance mechanisms to multiple groups of antibiotics. The synergy between an outer membrane with low permeability and efflux mechanisms is responsible for this intrinsic resistance. Against this background, this study was conducted to identify carbapenem resistance among *Pseudomonas* isolates through efflux-mediated mechanisms and assess the ability of efflux pump inhibitors to restore antibiotic sensitivity.[4]

Methods

This prospective cross-sectional study was carried out in a tertiary care teaching hospital from August 2021 to July 2022 after obtaining approval from the Institutional Ethical Committee (Tirunelveli Medical College ID: 1898/MICRO/2021). Clinical samples were collected from patients after obtaining written informed consent. The socio-demographic details of the patients were recorded. Appropriate samples were collected from patients based on their clinical manifestations under strict aseptic precautions.

Operational definition: Multidrug-resistant isolate is an isolate that is non-susceptible to at least one agent, in three or more antimicrobial categories.

Bacterial isolation and identification: A total of 50 non-duplicate multidrug-resistant *P. aeruginosa* isolates from various clinical samples, such as pus, blood, urine, sputum, and tissue from hospitalized patients, were collected. They were processed by conventional microbiological methods such as Gram staining, catalase test, oxidase test, and biochemical tests for identification.

Antimicrobial susceptibility testing: The Kirby-Bauer disk diffusion method on Mueller-Hinton agar was used to test antibiotic susceptibility in accordance with Clinical and Laboratory Standards

Institute (CLSI) standards. *Pseudomonas aeruginosa* ATCC 27853 was used as the standard control strain. The following antibiotics were tested: ceftriaxone, cefotaxime, ceftazidime, gentamicin, amikacin, ciprofloxacin, meropenem, piperacillin-tazobactam, and imipenem.

All *P. aeruginosa* isolates were tested for meropenem (10 µg) susceptibility. All meropenem-resistant strains were further tested for efflux-mediated resistance using the Carbonyl Cyanide 3-Chloro Phenylhydrazone (CCCP) phenotypic test (Figure 1).

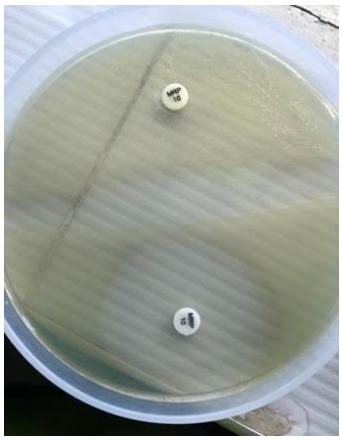

Figure 1. Carbonyl Cyanide 3-Chloro Phenylhydrazone (CCCP)

Detection of efflux pump activity by CCCP.[5] The MexE efflux pump inhibitor CCCP restores antibiotic action in bacteria that have become resistant due to overexpression of the MexE efflux pump. Mueller-Hinton agar plates comprising 12.5µM Carbonyl Cyanide 3-Chloro Phenylhydrazone (Sigma-Aldrich, Germany) were prepared. The isolates were adjusted to 0.5 McFarland standard and inoculated onto CCCP-supplemented Mueller-Hinton agar plates and simultaneously onto control plates without CCCP. Meropenem discs were placed on both plates and incubated at 37 ºC for 24 hours. A zone size of more than 19 mm around the meropenem disc on the CCCP-supplemented plate was considered positive for efflux pump activity, whereas a zone size of less than 15 mm around the meropenem disc was considered negative.

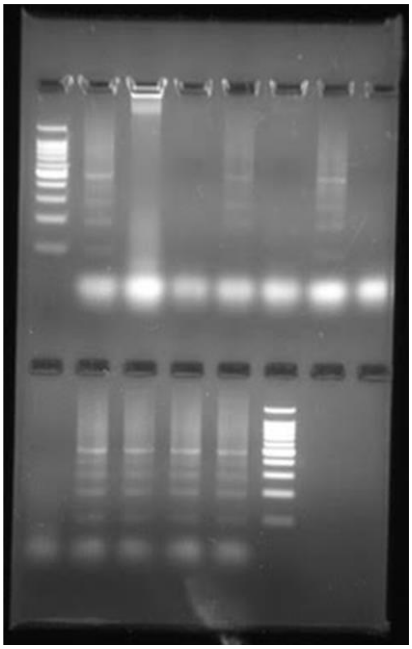

Figure 2. Gel electrophoresis showing positive for *mexE* gene (100 base pairs)

Genotypic assay: DNA extraction and polymerase chain reaction: The isolates were first inoculated in Luria-Bertani broth and incubated overnight, from which 2 ml was used for DNA extraction. DNA extraction was performed according to the kit manufacturer's guidelines (Helini Biomolecules, Chennai, India).

Detection of the *mexE* gene was performed using the forward primer 5'-GTCATCGAACAACCGCTG-3' and reverse primer 5'-GTCGAAGTAGGCGTAGACC-3', which produces an amplicon size of 516 bp. The efflux pump components are encoded by the *mexE* gene. PCR was performed using a conventional method with a thermocycler following the manufacturer's protocol.

Agarose gel electrophoresis was performed, and the newly synthesized DNA fragments of the *mexE* gene were verified by the development of bands according to their molecular weight using a 100 bp DNA ladder as reference (Figure 2).

Statistical Analysis

Microsoft Excel was used to enter the collected data, and SPSS version 25.0 (IBM Corporation, Armonk, NY, USA) was used for analysis. Continuous variables were shown as mean ± standard deviation, whereas categorical variables were recorded as numbers and percentages. Differences between qualitative variables were analysed using the Chi-square test. Statistical significance was set at $P < 0.05$.

Results

Of the 50 multidrug-resistant *P. aeruginosa* strains, 18 (36%) were isolated from pus, 13 (26%) from urine, 8 (16%) from blood, 6 (12%) from sputum, and 5 (10%) from endotracheal (ET) aspirates. Among these, 12 (24%) were from the General Surgery department, followed by 10 (20%) from General Medicine.

Among the 50 *P. aeruginosa* isolates tested with carbonyl cyanide m-chlorophenylhydrazone (CCCP), 12 (24%) exhibited phenotypic efflux-mediated resistance, while 38 (76%) showed resistance due to other mechanisms (Table 1).

Table 1: Distribution of study isolates between the phenotypic and genotypic assays

CCCP test	Conventional PCR		Total
	Positive	Negative	
Positive	7	5	12
Negative	0	38	38
Total	7	43	50

Sensitivity:100%, Specificity: 88%, Positive Predictive Value: 58.3%, Negative Predictive Value: 100%. Fisher's Exact test: <0.0001, Statistical association

Conventional PCR was used to identify the *mexE* gene in resistant *P. aeruginosa* isolates. Among the 50 isolates, the *mexE* gene was identified in 7 (14%), while 43 (86%) tested negative (Table 1).

Efflux-mediated resistance among the 50 *P. aeruginosa* isolates was assessed using both phenotypic and genotypic methods. Among the 12 (24%) isolates exhibiting phenotypic resistance via the CCCP method, only 7 (14%) were found to be *mexE*-positive by PCR.

Discussion

The Mex efflux pumps in *P. aeruginosa* are notable for their broad substrate specificity. They belong to the Resistance–Nodulation–Division (RND) superfamily, and their overexpression contributes to resistance against multiple classes of antibiotics. The genome of P. *aeruginosa* contains twelve efflux systems in this family, where four are widely described as antibiotic transporters (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM).[2,6,7] Although each efflux pump is primarily associated with certain antibiotic classes, fluoroquinolones are known to be common substrates for all four systems.

Several structurally diverse compounds have been evaluated for their inhibitory effects on Gram-negative bacterial efflux systems, including phenylalanine–arginine β-naphthylamide (PaβN), arylpiperazine derivatives, quinolones, and carbonyl cyanide m-chlorophenylhydrazone (CCCP).[8] In the present study, CCCP was used strictly as an *in vitro* laboratory reagent for phenotypic

detection of efflux pump activity and not as a therapeutic agent. Due to its toxic nature, CCCP has no role in clinical patient management.

Molecular diagnostic methods are increasingly replacing conventional culture-based and serological techniques due to their superior speed and accuracy. Reverse transcription PCR (RT-PCR), in particular, enables detection of gene expression levels and has been employed in identifying efflux pump activity in *P. aeruginosa*.[9] However, these molecular techniques do not directly measure the protein's quantity or functional activity. Therefore, combining phenotypic and genotypic approaches is advisable for accurately identifying Mex-mediated efflux resistance in *P. aeruginosa*.[2,10]

In the present study, among the 12 (24%) isolates that exhibited phenotypic efflux-mediated resistance via the CCCP method, only 7 (14%) were confirmed to carry the *mexE* gene using conventional PCR. The remaining 5 isolates that tested positive phenotypically but negative genotypically, may have exhibited resistance through other efflux systems or alternative mechanisms.

Efflux pump inhibitors (EPIs) have been investigated as an alternative strategy to combat antimicrobial resistance in *P. aeruginosa*, offering a promising adjunct to conventional antibiotic therapy. These agents act by increasing the intracellular concentration of antibiotics, thereby enhancing bacterial susceptibility. EPIs exert two major effects: they re-sensitize resistant organisms to existing antibiotics and reduce the emergence of resistant mutations. They can be derived from both natural and synthetic sources.[11] Thus, the discovery and development of effective EPIs for high-priority pathogens such as *P. aeruginosa* remain both urgent and significant.

This study has several limitations. It was conducted at a single centre with a relatively small sample size. Genotypic analysis was limited to conventional PCR, which may not detect other efflux pump genes contributing to multidrug resistance. Additionally, sequencing of the clinical isolates was not performed due to financial constraints.

Conclusion

Among the 50 multidrug-resistant (*MDR*) *P. aeruginosa* isolates, 12 (24%) exhibited phenotypic efflux-mediated resistance as determined by the CCCP method, and seven (14%) were positive for the *mexE* gene by conventional PCR, which contribute to multidrug resistance in the local setting, though they are not universally present among all MDR strains. Given that *P. aeruginosa* possesses both intrinsic and acquired resistance mechanisms against multiple classes of antibiotics, it remains a significant therapeutic challenge, particularly in hospitalized patients. These findings underscore the need for continuous surveillance and the formulation of institution-specific antibiotic stewardship policies to effectively manage and contain resistance.

Declarations

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Ethics statement: Ethical approval was obtained from the Institutional Ethics Committee of Tirunelveli Medical College, Tirunelveli, Tamil Nadu, India (reference number TIREC Protocol ID 1898/MICRO/2021)
Authors' contributions: Conception and design, acquisition of data, analysis and interpretation of data: AP, PSK, NR, IA. Drafting the article, revising it critically for important intellectual content and final approval of the version to be published: AP, PSK, NR, IA.
Data availability: The data supporting this study's findings are available from the corresponding author, PSK, upon reasonable request.
Disclaimer: The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy of

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