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Original Research Article

Study of antimicrobial resistance profile and efflux mediated drug resistance in clinical isolates of *Pseudomonas aeruginosa* detected by ethidium bromide-agar Cartwheel method

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ABSTRACT

Background: Development of antibiotic resistance or multidrug resistance (MDR) is one of the major causes of treatment failure of bacterial infections and has rapidly evolved into a threat to global health care. Bacterial efflux pumps play a significant role in the development of antibiotic resistance. This study evaluates the prevalence of efflux pump- mediated drug resistance in clinical isolates of *P. aeruginosa*.
Methods: Clinical isolates (n=100) of *P. aeruginosa* were collected from various clinical laboratories in Kerala and their efflux mediated drug resistance detected by the ethidium bromide (EB) agar Cartwheel method.
Results: The EB agar cartwheel method of screening revealed efflux activity in 17% (n=17) strains. The efflux activity was revealed at a minimum concentration of EB at 0 mg/l. *P. aeruginosa* strains showed the highest activity up to a concentration of 2.5 mg/l.
Conclusions: This study discusses the emergence of efflux pump- mediated drug resistance in *P. aeruginosa* from various clinical samples. Our results showed that 17% drug resistance in *P. aeruginosa* is attributable to efflux related mechanisms.

Keywords: Multidrug resistance, Efflux pumps, Ethidium bromide Cartwheel method

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous non fermentative gram-negative bacteria with extraordinary ability to survive on a wide variety of surfaces. It is a major cause of nosocomial infection in particular affecting patients with impaired immune systems and in patients admitted to intensive care.[1-5] It has been implicated in nosocomial urinary tract infection (UTI), ventilator associated pneumonia (VAP), ocular infections associated with contaminated contact lens solutions, ear infections, skin and soft tissue infections and rarely a cause of community acquired pneumonia, endocarditis and meningitis.[8] *P. aeruginosa* possess several virulence factors that actively aid in its pathogenicity and resistance to anti-microbial agents.[6] Development of antibiotic resistance is one of the main causes for treatment failure in microbial infections like *P. aeruginosa*.[7]

P. aeruginosa is often a cause of concern due to intrinsic resistance to many drug classes and the ability to acquire resistance by multiple mechanisms. This has led to an alarming increase in resistance particularly in serious nosocomial infections.[9] An increase in multidrug

resistance (MDR) and extensively drug resistance (XDR) *P. aeruginosa* with rates between 15 and 30% in some geographical areas has been reported in recent years.[10] *P. aeruginosa* can develop resistance to antibiotic classes either through the acquisition of resistant mobile genetic elements like plasmids or through mutational process that alter the expression and/or functions of chromosomally encoded mechanisms. Both strategies for developing drug resistance can severely limit the therapeutic options for treatment of serious infection.[11] Intrinsic resistance mechanisms of *P. aeruginosa* includes its low outer membrane permeability, presence of antibiotic efflux pumps and beta–lactamases. Acquired resistance mechanisms from horizontal gene transfer include acquisition of transferable antibiotic modifying enzymes. Mutational resistance leads to an over-expression of efflux pump and beta lactamases and a decreased expression of target sites.[12]

Efflux pumps play an important role in antibiotic resistance of *P. aeruginosa*.[13] This microorganism presents several mutative MDR efflux pump encoding genes belonging to the RND family of bacterial transporters.[14] *P. aeruginosa* us armed with a repertoire of at least 12 RND efflux pumps. Four of these namely, Mex –AB-Opr M, Mex CD-Opr J, MexEF-Opr N and Mex XY-Opr M are clinically relevant and have been implicated in the development of antibiotic resistance.[15] The aim of this study is to characterize antibiotic susceptibility and detect efflux pump mediated drug resistance in *P. aeruginosa*.

METHODS

The present cross-sectional study was conducted at School of Medical Education (SME), Kottayam, Kerala from January 2022 to March 2022. 100 isolates of *P. aeruginosa* collected from various diagnostic microbiology laboratories in Central Kerala during the study period was further identified and tested. Only clinically significant isolates of *P. aeruginosa* from urine, pus and exudates was included in the present study and all isolates of *P. aeruginosa* from other samples were excluded.

Identification of P. aeruginosa and antimicrobial susceptibility testing

All *P. aeruginosa* isolates were identified by routine biochemical testing and antimicrobial susceptibility testing by disc diffusion was performed as prescribed by Clinical Laboratory Standards Institute, M02-A13. The following antibiotics were tested; gentamicin (10 µg), amikacin (30 µg), imipenem (30 µg), meropenem (10 µg), ceftazidime (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), piperacillin-tazobactam (100/10 µg), cefaperazone-sulbactam (75/10 µg), ceftazidime-avibactam (50 µg), and aztreonam (30 µg). Based on the recommendations of the Centers for Disease control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC), isolates were termed MDR if they exhibited non susceptibility to at least one agent in three or more antimicrobial categories. All the reagents, culture media and antibiotic disc were obtained from HiMedia except ceftazidime – avibactam which was sourced from Thermo-Fisher.

Evaluation of efflux activity of P. aeruginosa – ethidium bromide-agar (Et-Br) Cartwheel method

Efflux activity was evaluated using ethidium bromide-agar Cartwheel method as prescribed by Martins et al with minor modifications.[16] Bacterial strains were grown in 5 ml trypticase soy broth (TSB) at 37^0C overnight. Turbidity was adjusted to 0.5 Mac-Farland standard. Trypticase soy agar (TSA) plates containing Ethidium Bromide concentration ranging from 0 mg/l to 2.5 mg/l were prepared on the same day of experiment and protected from light using zinc foils. The plates were divided in to four sectors by radial lines (Cartwheel pattern) as shown in the Figure 1 (one negative strain and 3 test strains). The turbidity adjusted cultures of test strains were swabbed on to ethidium bromide agar plates starting from center of the plate and spreading towards the edge as indicated by arrow heads shown in the Figure 1. Every test plate included a negative reference strain, *P. aeruginosa* MTCC 3541 as the comparative control for fluorescence. The inoculated ethidium bromide agar plates incubated overnight at 37^0C were examined under a UV transilluminator for fluorescence.[17]

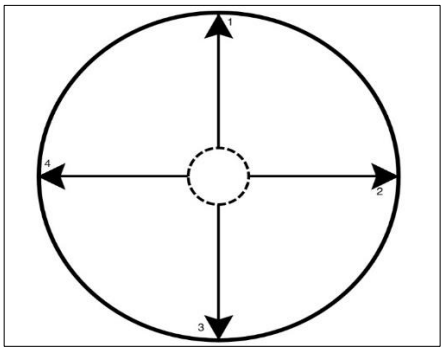

Figure 1: Cartwheel pattern for the swabbing of the bacterial strains tested by the EB agar cart wheel method. Controls and clinical isolates were swabbed on an EB containing agar plate, according to the diagram. This TSA-EB agar plate will accommodate four bacterial strains. Schematic representation of this petri plate of the figure denotes the position of bacterial strains (1) *P. aeruginosa* MTCC 3541 control strain, (2-4) *P. aeruginosa* MDR strains.

The study was approved by the institutional ethical committee at School of Medical Education. The data was analysed using Microsoft excel 2019 and statistical package for the social sciences (SPSS-18).

RESULTS

During the study, 100 *P. aeruginosa* were obtained from various samples-urine (n=36), pus (n=55), and exudates

(n=9). Of the 100 samples 56% (n=56) were obtained from males and 44% (n=44) from females. The study population was further sub-divided based on age as 0-7, 8-19, 20-39, 40-60 and >60. The age wise distribution of positive cultures was as follows: 10 (5 males, 5 females) for the 8-19 age group corresponding to 10% of the total isolates, 20 (10 males, 10 females) for the 20-39 age group, 40 (25 males, 15 females) for 40-60 age group and 30% (16 males, 14 females) for >60 age group (Figure 2).

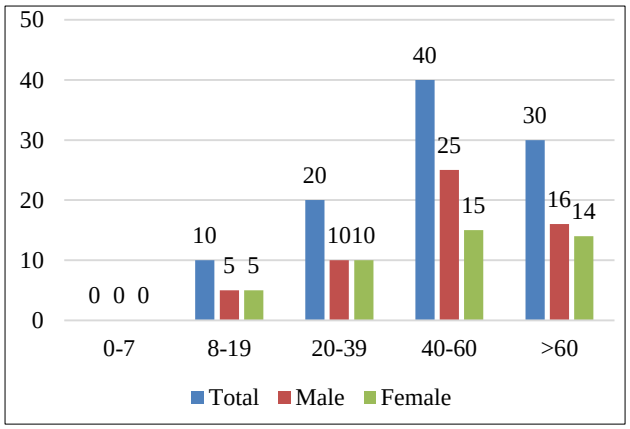

Figure 2: Age wise distribution of *P. aeruginosa* isolates.

The antibiogram of *P. aeruginosa* obtained in the present study is given in Table 1. Out of 100 clinical samples, 87% (n=87) were sensitive to Amikacin while 13% (n=13) exhibited resistance. 83% (n=83) displayed sensitivity, 16% (n=16) were resistant, and 1% (n=1) was classified as of intermediate susceptibility to gentamicin (GEN). 79% (n=79) of isolates were sensitive to aztreonam (AZ) while 8 samples were of intermediate susceptibility. The remaining 13% (n=13) were resistant. Ceftazidime (CAZ) resistance was observed in 16% of the isolates. 17% were resistant to cefepime (CPM). Cefaperazone-sulbactam (CFS) resistance was observed in 16% (n=16) of the isolates and 2% (n=2) were classified as intermediate. 16 % of isolates were resistant to ciprofloxacin (CIP), piperacillin-tazobactam (PIT), meropenem (MER) and imipenem (IMP). However, an intermediate susceptibility of 1% (n=1) by isolates to ciprofloxacin was also observed. 85% (n=85) of the isolates were sensitive to ceftazidime-avibactam (CZA) while the remainder (15%) were shown to be resistant. Of the 100 *P. aeruginosa* strains isolated, 16% (n=16) were classified as MDR.

Efflux activity by ethidium bromide (Et-Br) agar Cartwheel method was detected in 17% (n=17) of the total isolates i.e. no fluorescence was produced. Six isolates at a concentration of 0.5 mg/l, four at 1 mg/l, four at 1.5 mg/l, two at 2 mg/l and one at 2.5 mg/l were found to be positive for efflux activity. MTCC 3541 *P. aeruginosa* taken as a control strain shows fluorescence in all concentrations from 0.5 mg/l to 2.5 mg/l (Figure 3).

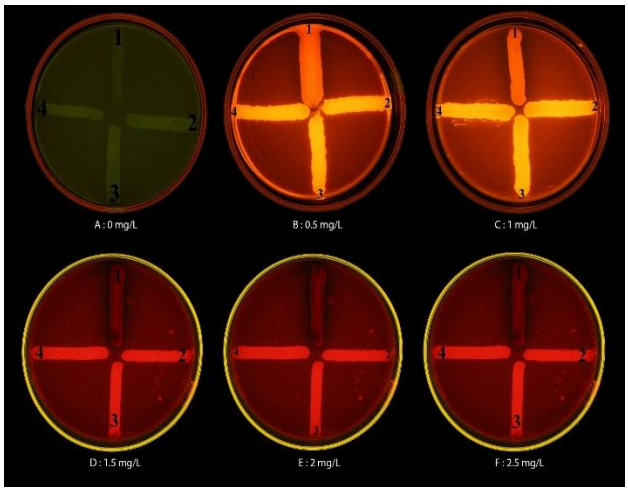

Figure 3: Accumulation of fluorescent chromophore – trypticase soy agar plates containing varying concentrations of ethidium bromide, swabbed with *P. aeruginosa* strains. Control strain (1) shows fluorescence from A (0 mg/l) to F (2.5 mg/l). Control strain (1) and MDR strains (2-4) shows minimum fluorescence at 0.5 mg/l higher up to 2.5 mg/l (B-F). All strains do not produce fluorescence at 0 mg/l (A).

Table 1: Antimicrobial susceptibility of *P. aeruginosa* isolates to different antibiotics.

Antibiotic	Code	Sensitive		Resistant		Intermediate		Total
		Frequency	Percent	Frequency	Percent	Frequency	Percent	
Amikacin	AK	87	87	13	13	0	0	100
Gentamicin	GEN	83	83	16	16	1	1	100
Aztreonam	AZ	79	79	13	13	8	8	100
Ceftazidime	CAZ	84	84	16	16	0	0	100
Cefepime	CPM	83	83	17	17	0	0	100
Cefoperazone sulbactam	CFS	82	82	16	16	2	2	100
Ciprofloxacin	CIP	83	83	16	16	1	1	100
Piperacillin-tazobactum	PIT	84	84	16	16	0	0	100
Meropenem	MRP	84	84	16	16	0	0	100
Imipenem	IMP	84	84	16	16	0	0	100
Ceftazidime-avibactam	CZA	85	85	15	15	0	0	100

DISCUSSION

Managing *P. aeruginosa* infection in Asia, Pacific is particularly challenging due to an increased resistance to antimicrobials. The rapid increase of drug resistant *P. aeruginosa* infection has been found to significantly burden the management of health care in hospital settings.[18] In addition to intrinsic resistance against many classes of antimicrobials, *P. aeruginosa* has acquired multiple mechanisms of resistance. Over-expression of efflux pump is one such acquired mechanism. The role of efflux pump over-expression contributing to resistance is difficult to infer based on susceptibility patterns and must be systematically examined.[19]

The present study revealed antibiotic resistance ranging from 13 to 17% with amikacin exhibiting the least resistance and cefepime, the most. Various studies have also reported similar resistance patterns (4.4 % to 17.8%, 6.4% to 21.3%).[20,21] However, some studies reported higher resistance patterns ranging from 17% to 87%, 8.4% to 39.39%, 6 to 48% of the isolates.[22-24] This disparity in resistance could be explained by differences in sample size, and distribution of various specimens.

In the present study, though resistance percentages were between 13 and 17, MDR organisms were at 16% (n=16) of the total isolates.[25] A study by Rejani et al also reported 12.21% (n=37) of strains to be MDR. The difference in the study by Rejani et al from our study might, in part, draw from the larger sample size (n=256).

The current study was to detected efflux mediated drug resistance in *P. aeruginosa* that cannot be detected by routine disk diffusion methods. The identification of efflux mediated MDR clinical isolates is labour intensive and requires specialized instruments such as fluorometers, flow cytometers or radioactivity detectors.[26-28] Other methods currently used for evaluation is a combination of the drug with an efflux pump inhibitor, which evaluates reduction in MIC of a given antibiotic when an efflux pump inhibitor is present in the medium.[29] This can be quite expensive and, therefore, may not be feasible in all laboratories.

We employed the ethidium bromide agar cartwheel method as it is a simple, instrument free method that uses agar plates containing increasing concentrations of ethidium bromide and can be easily adapted by a routine clinical microbiology laboratory.[30] The ethidium bromide agar cartwheel method has the advantage that multiple strains can be tested on the same agar plates.

Our study has limitations as only a single species of pathogen was tested and the sample size (n=100) is small. So, study with additional pathogens may provide better results. The reported data above require genotypic confirmation and further studies have been planned to investigate these isolates.

CONCLUSION

In conclusion, the detection of efflux activity by ethidium bromide agar Cartwheel method may prove to be a useful and economical tool in the detection of clinically relevant MDR phenotypes of *P. aeruginosa* that utilize the efflux pump mechanism because of the failure of conventional antimicrobial sensitivity testing to do so. The activity of these efflux mechanisms needs to be recognized early as drug resistance rapidly develops in hospitalized patients undergoing treatment and corrective measures benefit the adjustment of therapeutic strategies that minimize the selection of resistant variants.

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