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Research Article

Determination of Drug Resistance Mechanism (s) of Clinical Isolates of *P. aeruginosa* and Phytoextract as Drug Resistance Reversal Agent

Gaurav Raj Dwivedi[1,4]*, Avinash Singh[2], Harish C Upadhyay[3], Sanghmitra Pati[4], Devendra P Singh[1], Kashi N Prasad[2], Mahendra P Darokar[5] and Santosh K Srivastava[3]

[1]*Department and School of Environmental Science, Babasaheb Bhimrao Ambedkar Central University, Vidya Vihar, Lucknow, India*

[2]*Microbiology Department, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India*

[3]*Medicinal Chemistry Department, Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India*

[4]*Microbiology Department, ICMR-Regional Medical Research Centre Bhubaneswar, Bhubaneswar, Odisha, India*

[5]*Molecular Bioprospection Department, Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India*

***Corresponding Author**: Gaurav Raj Dwivedi, Microbiology Department, ICMR-Regional Medical Research Centre Bhubaneswar, Bhubaneswar, Odisha, India.

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Abstract

The deficiency of novel antibiotics in drug discovery pipeline and spreading out of drug resistance in gram negative bacteria placed human society on the edge of preantibiotic era. As a part of this project, the clinical isolates of *Pseudomonas aeruginosa* were assessed to find out the major mechanism of multidrug resistance (MDR). In this study, the clinical isolates were procured from Sanjay Gandhi Post graduate Institute of Medical Sciences (SGPGIMS), Lucknow. Sensitivity/drug resistance profiling of these clinical isolates of *P. aeruginosa* was done by broth dilution assay. The mechanism of extended spectrum beta lactamase (ESBL) and carbapenem resistance was done by ESBL and metallo beta lactamase (MBL) detection kits, while checkerboard assay with efflux pump inhibitors was done to find out the efflux pump mediated drug resistance. Efflux mediated drug resistance was the key mechanism in all the isolates of *P. aeruginosa*. Our findings will be helpful to find out the key mechanism of drug resistance as well as plant extract as drug resistance reversal agents either by inhibiting efflux pumps/MBL/biofilms.

Keywords: *Gram Negative Bacteria; MDR; Efflux Pumps; ESBL; Metallo Beta Lactamase; Plant Extract*

Abbreviations

MDR: Multidrug Resistance; MHA, MHB: Mueller Hinton Agar and Broth; WHO: World Health Organization; ESBL: Extended Spectrum Betalactamase; MBL: Metallo Betalactamase; EPI: Efflux Pump Inhibitors; FDA: Food and Drug Administration; IDSA: Infectious Diseases Society of America

Introduction

It is true that infectious diseases are still the major causes of morbidity and mortality in human beings even after the discovery and development of various anti-infective agents [1,2]. In the same way the discovery of and the development of antibiotics have not only transformed modern medicine but also have proven exceptionally useful in human health [3]. Multidrug resistant organisms (MDRO) not only put an additional burden of infection, but also result in inferior treatment by the antibiotics of latest generation [4]. On other hand, MDRO increases the severity of infections both in the hospital and community [5]. The mechanism behind the multidrug resistance phenomenon is often associated with over-expression of the efflux pumps which recognize and efficiently expel broad range of structurally unrelated drugs from the cells. Gram negative bacteria (GNB) exploits these efflux pumps to achieve high degree of resistance in coordination with outer membrane barriers and biofilms [6,7] and directly or indirectly responsible for the development of superbugs because these pumps not only make the antibiotics inefficient but also narrow down treatment option against GNB [3,8]. Efflux pump proteins are known as the

gatekeepers of cell and found to be involved in neurotransmission, sensing, and transport of nutrients and drugs into and out of cells [9]. In addition these proteins also provide promising targets for ~50% of all marketed pharmaceutical drugs [9,10].

P. aeruginosa is well characterized gram negative bacteria and is being used as a model organism for numerous biochemical, microbiological, and biotechnological studies [11,12]. *P. aeruginosa* is also leading causes of complications in cystic fibrosis which resulted in significant morbidity and mortality [13]. These peculiar qualities made multidrug-resistant (MDR) or extensively drug-resistant (XDR) *P. aeruginosa* strains a international problem [7,13]. Carbapenems supposed to be the best treatment options for MDR *P. aeruginosa* but the origin of different type of carbapenemases now makes the drug discovery more challenging against these superbugs [14-16]. In post-genomic era, these efflux pumps offer the "key" targets for the development of novel anti-bacterial agents [17-19].

Efflux pumps now became an established target while genus *Ammannia* are frequently used in traditional Chinese and Indian medicine [19,20]. However different species of *Ammannia* were already reported for numerous medicinal usages, but *A. baccifera* Linn. and *A. multiflora* is widely used in traditional Chinese and Indian herbal formulations for the treatment of a number of diseases including spinal disease, haemorrhoids, common cold, human female infertility and gastroenteropathy as well as anticancer, antirheumatic, antidiuretic, antipyretic, anti-steroidogenic, antimicrobial etc. activities [20,21]. In an earlier study, we already reported bio-enhancing potential of extracts and the compounds isolated from *A. multiflora*, wherein the 4-hydroxy-α-tetralone and its various semi-synthetic acyl and aryl derivatives showed bio-enhancing potential against the nalidixic acid sensitive and nalidixic acid resistant strains of *E. coli* [20,22].

In view of above, present study was planned to assess the key mechanism of MDR in the clinical isolates of *P. aeruginosa*. On the basis of resistance profiling ten clinical isolates were found to have resistance to two or more than two structurally unrelated antibiotics. In the backdrop of mechanism of action of MDR these clinical isolates were also assessed to know the major resistance mechanism mediated by either efflux pumps or ESBL and or metallo beta lactamase. The resistance was mainly mediated by drug efflux pumps in all the clinical isolates. In another study different extracts of *A. multiflora* was also evaluated as drug resistance reversal agents against the most resistant clinical isolate of *P. aeruginosa*.

Materials and Methods

Used bacterial cultures and media

A total 10 clinical isolates of *P. aeruginosa* coded as PG-1 to PG-10 were obtained from the Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India. One most resistant clinical isolate (PG10) was also used for the drug resistance reversal potential of plant extract [23]. Drug-sensitive strain of *P. aeruginosa* MTCC 741/ATCC 25668 was procured from Microbial Type Culture Collection, Chandigarh India. Standard Mueller Hinton agar and broth (MHA and MHB, Hi-Media, Mumbai, India) were used as bacterial culture media. Mueller Hinton broth no. 2 (control cations) was used for combination study. Colony counts were determined using MHA plates.

Susceptibility tests of antibiotics and plant extracts

The minimum inhibitory concentrations (MICs) were determined in Mueller Hinton broth using 96-well micro titer plates following the Clinical and Laboratory Standards Institute guidelines for broth micro dilution following the CLSI guidelines for broth micro-dilution [24,25]. Antibiotics namely streptomycin (STR), tobramycin (TOB), norfloxacin (NOR), ampicillin (AMP), erythromycin (ERY), tetracycline (TET), colistin (COL), polymyxin-B (POL), imipenem (IMP) and ethidium bromide (ETBR) were diluted into final concentrations of 1600 to 3.125 µg/mL and tested against *P. aeruginosa* isolates. The MIC values were determined by 2 fold serial dilution broth assay with starting inoculums of 5 x 10^5 cfu/mL. Inoculated plates were incubated at 37°C for 18 h, and visual observations were recorded as per CLSI guidelines. The susceptibility test of plant extracts was also determined by MIC determination. The details of phytoextracts used in this study are given in table 1.

Plant code	Plant Name	Parts used	Used solvents
AML-M	*Ammannia multiflora*	Leaves	Methanol
AMR-M	*A. multiflora*	Root	Methanol
AMS-M	*A. multiflora*	Stem	Methanol

Table 1: *Detail of plant extracts used in the study.*

ESBL Detection

Phenotypic ESBL detection was done by triple ESBL detection Ezy MICTM Strip (MIX+/MIX) (Himedia laboratories, Mumbai, India). It is a unique Phenotypic ESBL detection strip which is coated with mixture of 3 different antibiotics with and without clavulanic acid on a single strip in a concentration gradient manner. The upper half has Ceftazidime, Cefotaxime and Cefepime (Mixture) + Clavulanic acid with highest concentration tapering downwards, whereas lower half is similarly coated with Ceftazidime, Cefotaxime and Cefepime (Mixture) in a concentration gradient in reverse direction. Overnight grown culture of clinical isolate of *P. aeruginosa* with of standard 0.5 McFarland were plated on MHA plate and Ezy MICTM Strip (MIX+/MIX) was placed on the centre of the plate gently. Results were recorded in terms of ratio of values. When the ratio of the value obtained for MIX: the value of MIX in combination with Clavulanic acid (MIX+) is more than or equal to 8 it is positive if less than 8 negative if there is no zone of inhibition is obtained on either side following the CLSI guidelines [24]. In such cases resistance may be due to mechanisms other than ESBL production.

MBL detection

Phenotypic MBL detection was done by Imipenem with and without EDTA Ezy MIC™ Strips (Himedia laboratories, Mumbai, India). It is an unique phenotypic MBL detection strip which is coated with mixture of Imipenem+EDTA and Imipenem on a single strip in a concentration gradient manner. The upper half has Imipenem+ EDTA with highest concentration tapering downwards, whereas lower half is similarly coated with Imipenem in a concentration gradient in reverse direction. Overnight grown culture of clinical isolate of *P. aeruginosa* with of standard 0.5 McFarland were plated on MHA plate and Imipenem with and without EDTA Ezy MIC™ Strip was placed on the centre of the plate gently. Results were recorded in terms of ratio of values. When the ratio of the value obtained for Imipenem (IPM): the value of Imipenem + EDTA (IPM+EDTA) is more than to 8 it is positive if less than 8 negative if there is no zone of inhibition is obtained on either side following the CLSI guidelines.

***In vitro* combination studies**

Combination study was performed by the broth checkerboard method as described by Eliopoulos and Wennersten, 2002 [26]. Cation-adjusted Mueller Hinton broth (150 µL) was added to each well of the 96-well plate. The last four columns of wells served as controls for *P. aeruginosa* growth and plate sterility. The final concentrations ranged from 12.5 - 1600 µg/mL for efflux pump substrate antibiotics and from 0.78 - 100 µg/mL for known efflux pump inhibitors/plant extracts. Thus, each of the 64 wells had unique combinations of antibiotics and test compounds/extract. The final bacterial inoculum in each well was 5×10^5 cfu/mL except the negative controls. The plates were incubated at 37°C for 24h. The MIC was recorded as the last dilution without any turbidity as per CLSI guidelines. Results were recorded in terms of fold reduction.

Results and Discussion

Innovation gap explains the lack of novel structural classes introduced to the antibacterial armamentarium since decades, which was broken in 2015 by discovery of teixobactin, a new group of antibiotic shown to be effective against multidrug resistance gram positive bacteria [27,28]. There has been an enormous hope from clinicians, scientific and the public agency for a novel class of antibiotic but till date there is no new antibiotic against MDR-gram negative bacteria [3,15,23,28]. However hard work of the agencies such as FDA, Infectious Diseases Society of America (IDSA), and the European Medicines Agency(EPA) may lead in identifying novel antibiotics in coming future [13,29,30].

A total of 10 clinical isolates of *P. aeruginosa* were screened against nine different antibiotics and ethidium bromide. It was found that all clinical isolates were showing the resistance against two or more than two different group of antibiotics and ETBR (Table 2). The clinical isolates of *P. aeruginosa* (PG-1 to PG-10) were behaving as MDR. As evident from broth dilution assay that all the above isolates were resistant to ampicillin, erythromycin, streptomycin and tetracycline. As per CLSI drug resistance breakpoints, all the isolates were sensitive to imipenem [31] (Table 2). It is also evident from table2 that the clinical isolate PG-10 was most resistant isolate.

Clinical isolates of *P. aeruginosa*	Minimum inhibitory concentration (µg/mL) of different group of antibiotics/ETBR									
	STR	TOB	NOR	AMP	ERY	TET	COL	PB	IMI	ETBR
PG1	400	100	50	800	800	400	3.12	6.25	< 3.12	100
PG2	400	100	100	800	800	400	3.12	6.25	< 3.12	100
PG3	200	50	50	800	800	400	3.12	6.25	< 3.12	50
PG4	400	100	50	800	400	100	3.12	6.25	< 3.12	50
PG5	200	50	100	800	200	200	6.25	6.25	< 3.12	100
PG6	400	50	100	800	400	200	12.5	6.25	< 3.12	100
PG7	100	100	50	800	400	400	6.25	6.25	< 3.12	100
PG8	100	50	25	800	400	200	3.12	6.25	< 3.12	100
PG9	100	50	100	400	200	200	3.12	6.25	< 3.12	100
PG10	800	200	100	1600	1600	800	12.5	6.25	< 3.12	200

Table 2: *Antibiotic resistance/sensitivity pattern of different clinical isoltes of P. aeruginosa.*

ESBLs are those enzymes that mediate resistance to third generation cephalosporins (e.g. Ceftazidime, Cefotaxime, and Ceftriaxone) and monobactams (e.g. Aztreonam) and these antibiotics are called as extended-spectrum antibiotics. ESBL do not affect either cephamycins (e.g. Cefoxitin and Cefotetan) or carbapenems (e.g. Meropenem or Imipenem) [13]. In phenotypic ESBL detection, no zone of inhibition is obtained on either side in all the clinical isolates of *P. aeruginosa*. On the basis of observation it was predicted that in such cases resistance was due to mechanisms other than ESBL production [32]. The presence of an ESBL-producing organism in a clinical infection can result in treatment failure if one of the above classes of drugs is used [31].

The introduction of carbapenems into clinical practice was miracle for the treatment of serious bacterial infections caused by beta-lactam resistant bacteria [14,30]. Broad spectrum activity and stability to hydrolysis by most beta-lactamases, the carbapenems now have been the drug of choice for treatment of infections caused by penicillin-or cephalosporin resistant Gram-negative bacilli especially, extended spectrum β-lactamase (ESBL) producing Gram-negative infections [16]. The carbapenems namely imipenem and meropenem available for use in India [13,15]. In the present MBL detection study, the ratio of the value obtained for Imipenem (IPM): the value of Imipenem + EDTA (IPM+EDTA) was found less than 8. So it was negative for MBL mediated resistance which supported our antibacterial screening results where all the clinical isolates of *P. aeruginosa* were sensitive to imipenem.

In present studies, it is suggested that all the clinical isolates were resistant toward those clinically used antibiotics, which were structurally and functionally different. Interestingly, all were sensitive to imipenem, which is not a substrate of efflux pump and is resistant to all other antibiotics that are the substrate for one or the other efflux pumps. This indicates that efflux pump mediated drug resistance in these clinical isolates and thus making it suitable for drug discovery studies. In case of isolate PG-10 was the most resistant isolate to all the antibiotics those are substrate of any one of efflux pumps. It is deciphered that this isolate has the high level of MDR activity due to the efflux pumps while sensitivity towards imipenem describes itself no involvement of metallo-β-lactamase mediated resistance (Figure 1, Table 3) [11,16].

EPI ↓	Antibiotics→	STR	TOB	NOR	AMP	ERY	TET	COL	PB	IMI	ETBR
	MIC Alone →	800	200	100	1600	1600	800	12.5	6.25	< 3.12	200
	↓	MIC in combinations µg/mL (compounds/antibiotics)									
		STR	TOB	NOR	AMP	ERY	TET	COL	PB	IMI	EtBr
PAβN	800	50/50	50/12.5	50/25	25/100	50/100	50/50	ND	ND	ND	12.5/25
RES	1600	50/400	100/100	50/100	100/800	50/1600	25/400	ND	ND	ND	100/100
AML-M	1600	50/200	50/50	100/50	50/800	50/800	50/200	ND	ND	ND	50/50
AMR-M	1600	50/400	50/100	100/50	100/800	100/800	50/400	ND	ND	ND	100/100
AMS-M	1600	50/400	100/100	100/50	100/800	100/800	50/400	ND	ND	ND	100/100

Table 3: *MIC of antibiotics in combination with efflux pump inhibitors/plant extracts against PG10 clinical isolate of P. aeruginosa.*

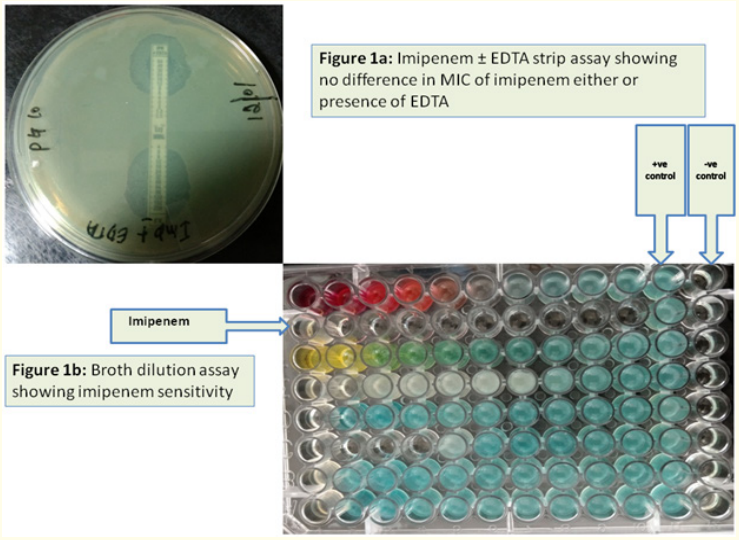

Figure 1: *Profiling of clinical isolate of P. aeruginosa (PG-10) (a) Imipenem EDTA assay (b) Broth dilution assay.*

The association of β-lactamases with over expression of efflux pumps and/or porin loss may lead to the emergence of high level resistance phenotypes [4,16,23]. For this reason, efflux pumps may seriously impact antimicrobial therapy in clinical settings. New agents with novel mechanisms of action are limited and it emphasizes the need for the development of new drugs/drug targets. Thus along with known efflux pump inhibitors, methanolic extracts of *A. multiflora* were evaluated for antibacterial and drug resistance reversal potentials. It is evident from result that individually these extracts did not show any antibacterial activity alone but in combination with antibiotics these were able to reduce the MICs of antibiotics up to four folds. This study provides evidence that the *A. multiflora* leaf methanolic extract possess significant drug resistant reversal activity against the drug-resistant isolate of *P. aeruginosa*. This extract was even better than a known drug resistance reversal agent reserpine. However, further studies are needed to confirm the corresponding mechanisms of action.

Conclusion

This work was planned to study the key mechanisms of resistance among *P. aeruginosa* clinical isolates either by production of ESBL, metallo-β-lactamases, or by operation of efflux systems or both. The above insights would ultimately help the physician in early detection of MDR strains from clinical specimens so that the appropriate antibiotic therapy can be initiated to have better clinical outcome.

While drug resistance reversal potential of plant extract may be helpful in the management of MDR mechanism by (i) lowering the dose of antibiotics; (ii) reducing the drug resistance development frequency; and (iii) increasing the efficacy of antibiotics against multidrug-resistant *P. aeruginosa*. These results may be of great help in standardized antibacterial extract formulations from a very common, inexpensive, and nontoxic natural product.

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Conflict of Interest

The author(s) confirm that this article content has no conflict of interest.

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