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Determination of MDR Mechanisms of *P. aeruginosa* Clinical Isolates

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Abstract

The lack of novel antibiotics and development of different level of drug resistance in gram negative bacteria put our civilization on the verge of preantibiotic era. In post genomic era drug resistant genes/proteins may be used as promising drug targets. As a part of our drug discovery program, the clinical isolates of *Pseudomonas aeruginosa* were explored to find major mechanism of multidrug resistance (MDR). In this study, the clinical isolates were procured from King George Medical University, Lucknow. Drug resistance profiling of these clinical isolates of *P. aeruginosa* was done by broth dilution assay. Extended spectrum betalactamase (ESBL) and metallo betalactamse (MBL) production was also done. After the checkerboard assay with efflux pump inhibitors, it was also found that the major mechanism of MDR was due to drug efflux pumps. One clinical isolate KG-P2, was found the most resistant isolate where the high level of multidrug resistance was governed by efflux pumps and MBL. Our findings suggest that drug efflux may be common of mechanism of MDR which will be helpful in identification and screening of natural compounds as efflux pump inhibitors (EPI)/MBL ihibitors.

Keywords: *Drug Resistance; Clinical Isolate, Pseudomonas aeruginosa; Efflux Pumps, ESBL, Metallo Beta Lactamase*

Abbreviations

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

Introduction

Even after the discovery and development of various anti-infective agents, infectious diseases are still the major causes of morbidity and mortality in human beings. Bacteria, viruses, parasites and fungi have affected human health and economy [1,2]. Innovation gap describes the lack of novel structural classes introduced to the antibacterial armamentarium since 1962. The long gap was broken in 2015 by discovery of teixobactin, a new group of antibiotic shown to be effective against multidrug resistance gram positive bacteria but these is no new antibiotics for the gram negative bacteria [3]. The multidrug resistance phenomenon is often associated with over-expression of the transporters that recognize and efficiently expel broad range of structurally unrelated compounds from the cells. Multidrug resistant organisms put an additional burden of infection, which ultimately result in inferior treatment by the antibiotics of latest generation

[4]. On other hand, MDR organisms increase the severity of infections both in the hospital and community [5]. *Pseudomonas aeruginosa* is one of the leading causes of complications in cystic fibrosis in humans [6]. Significant morbidity and mortality is often associated with multidrug-resistant (MDR) or extensively drug-resistant (XDR) *P. aeruginosa* strains [6]. MDR and or XDR *P. aeruginosa* have now become a international problem [7,8]. In prokaryotic system, efflux system was first described as a mechanism of resistance to tetracycline in *Escherichia coli* [9]. Gram negative bacteria (GNB) exploits efflux pumps to achieve high degree of resistance in coordination with outer membrane barriers and biofilms [8,10]. Efflux pumps are now known as a major reason of multidrug resistance rendering existing antibiotics ineffective and insisting for new therapeutic options [11]. These efflux pumps are 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 [12]. *P. aeruginosa* is one of the well characterized gram negative bacteria and is being used as a model organism for numerous biochemical, microbiological, and biotechnological studies [13,14]. These membrane proteins are known as the gatekeepers of cell and have been involved in neurotransmission, sensing, and transport of nutrients and drugs into and out of cells. In addition these proteins also provide promising targets for ~50% of all marketed pharmaceutical drugs [15]. Carbapenems were earlier supposed to be the best treatment options for MDR but the origin of different type of cabapenemases now makes the drug discovery more challenging against these superbugs [16,17]. In post-genomic era, these efflux pumps offer the "key" targets for the development of novel anti bacterial agents [18,19].

In view of above, present study was planned to explore 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 explored 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 nine clinical isolates while an isolate was resistant to imipenem also where the resistance was regulated by efflux pumps and metallo beta lactamases.

Materials and Methods

Used bacterial cultures and media

A total 110 clinical isolates *P. aeruginosa* clinical isolates coded as KG-P1 to KG-P110 were obtained from the Department of Microbiology, King George Medical University Lucknow, India. 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

The minimum inhibitory concentrations (MICs) were determined in Mueller Hinton broth using 96-well microtiter plates following the Clinical and Laboratory Standards Institute guidelines for broth microdilution following the CLSI guidelines for broth micro-dilution [22]and [23]. Antibiotics namely streptomycin (STR), tobramycin (TOB), norfloxacin (NOR), ampicillin (AMP), erythromycin (ERY), tetracycline (TET), colistin (COL), polimyxin-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* strains. The MIC values were determined by 2 fold serial dilution broth assay with starting inoculums of $5x10^5$ cfu/mL. Inoculated plates were incubated at 37°C for 18 h, and visual observations were recorded as per CLSI guidelines.

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 & 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 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 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 [22]. In such cases resistance may be due to mechanisms other than ESBL production.

MBL detection

Phenotypic MBL detection was done by Imipenem with & 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 [24]. 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 to 1600 µg/mL for efflux pump substrate antibiotics and from 0.78 to 100 µg/mL for imipenem as well as known efflux pump inhibitors. Thus, each of the 64 wells had unique combinations of antibiotics and test compounds. 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

Teixobactin is an antibiotic of a novel class produced by an undescribed soil microorganism (provisionally named *Eleftheria terrae*) was isolated by the iChip technology which permitted the environmental bacterium to grow, produce this antibiotic and subsequently identified [3]. However, teixobactin was reported to have activity against gram positive including mycobacteria but not gram negative bacteria with novel mode of action [25]. There has been an immense hope from scientific, clinicians and the public for a novel class of natural product such as antibiotic but till date there is no new antibiotic against MDR-gram negative bacteria [3,16]. However agencies such as FDA, Infectious Diseases Society of America (IDSA), and the European Medicines Agency may lead in identifying new antibiotics in coming future [6,26,27].

A total of 110 clinical isolates of *P. aeruginosa* were screened against 8 different groups of antibiotics and ethidium bromide. It was found that only 10 clinical isolates were showing the resistance against two or more than two different group of antibiotics and ETBR the remaining strains are screened out (Table 1). These isolates KG-P1 to KG-P10 isolates of *P. aeruginosa* were the MDR. As evident from broth dilution assay it was found that all the above isolates were resistant to ampicillin, erythromycin, streptomycin and tetracycline. As per CLSI drug resistance breakpoints, nine isolates were sensitive to imipenem while an isolate KG-P2 was found to resistant to imipenem [28] (Table 1, Figure 1).

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
KG-P1	200	3.12	3.12	1600	800	200	< 3.12	< 3.12	< 3.12	400
KG-P2	1600	100	100	> 1600	1600	1600	< 3.12	< 3.12	12.5	1600
KG-P3	200	50	50	1600	800	400	< 3.12	< 3.12	< 3.12	400
KG-P4	100	< 3.12	< 3.12	800	400	100	< 3.12	< 3.12	< 3.12	200
KG-P5	1600	100	100	1600	800	400	< 3.12	< 3.12	< 3.12	400
KG-P6	800	100	100	800	200	200	< 3.12	< 3.12	< 3.12	400
KG-P7	50	6.25	25	800	800	200	< 3.12	< 3.12	< 3.12	200
KG-P8	50	3.12	< 3.12	800	400	200	< 3.12	< 3.12	< 3.12	200
KG-P9	50	< 3.12	< 3.12	400	200	100	< 3.12	< 3.12	< 3.12	200
KG-P10	400	100	50	400	200	400	< 3.12	< 3.12	< 3.12	200

Table 1: *Antibiotic resistance/sensitivity pattern of different clinical isoltes of P. aeruginosa.*

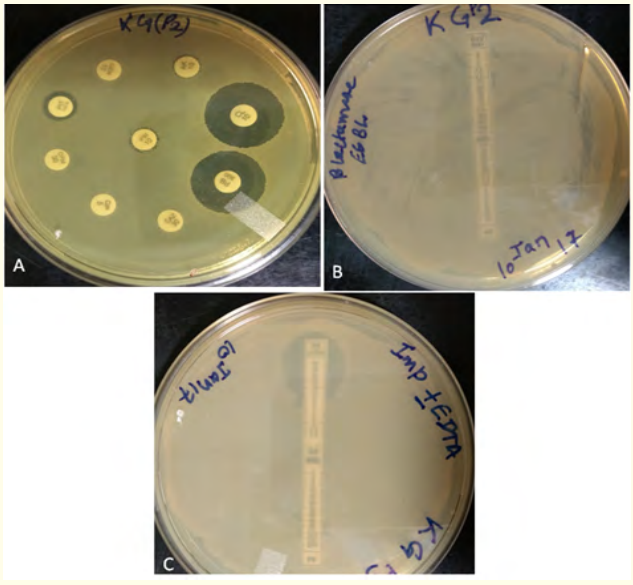

Figure 1: *Profiling of clinical isolate of P. aeruginosa (KG-P2) (A) Drug resistance/sensitivity profile (B) ESBL E test (C) MBL E test.*

In phenotypic ESBL detection, no zone of inhibition is obtained on either side in all the clinical isolates of *P. aeruginosa* (Figure 1). In such cases resistance may be due to mechanisms other than ESBL production [29]. 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., Cephoxitin and Cefotetan) or carbapenems (e.g., Meropenem or Imipenem) [18]. 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 [28]. ESBLs can be difficult to detect because they have different levels of activity against various cephalosporins. Thus, the choice of which antimicrobial agents to test is critical.

In case of isolate KG-P2, which was the most resistant isolate to all the antibiotics those are substrate of any one of efflux pumps as well as imipenem. It is deciphered that this isolate has the high level of MDR activity due to the coordination of efflux pumps with metallo betalactamase (Figure1, Table 2). The introduction of carbapenems into clinical practice was miracle for the treatment of serious bacterial infections caused by beta-lactam resistant bacteria [7]. 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 [17]. The carbapenems namely imipenem and meropenem available for use in India [18,30].

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

Table 2: *MIC of antibiotics in combination with efflux pump inhibitors against KG-P2.*

Even carbapenem resistance has been observed frequently in non fermenting bacilli *P. aeruginosa* and *Acinetobacter* spp [13,17]. In present studies, antibiogram suggested that these nine clinical isolates were resistant toward those clinically used antibiotics, which were structurally and functionally different. Interestingly, nine 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 nine clinical isolates and thus making it suitable for drug discovery studies. 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 [4,16,30]. For this reason, efflux pumps may seriously impact antimicrobial therapy in clinical settings. The supply of new agents with novel mechanisms of action is limited and it emphasizes the need for the development of new drugs/drug targets.

Conclusion

In India, we do not have any reports whether the major resistance mechanisms are operative either in isolation or in combination among the clinical isolates *P. aeruginosa*. Therefore 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. Further the study is also planned to screen the potent inhibitors from different resources to combat the major resistance mechanisms.

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Conflict of Interest

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

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