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Rising Antimicrobial Resistance of *Pseudomonas aeruginosa* Isolated from Clinical Specimens in India

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Abstract

The rising antibiotic resistance against commonly used drugs is of great concern. Drug susceptibility testing and Polymerase Chain Reaction (PCR) assay were used to determine the antibiotic susceptibility patterns and prevalence of genes encoding extended-spectrum-β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) among 515 isolates of *Pseudomonas aeruginosa* isolated from various clinical specimens. Susceptibility of isolates to seven antibiotics was tested using disc diffusion method according to the guidelines defined by Clinical Laboratory Standard Institute. Isolates showing resistance to any of the two cephalosporins (ceftriaxone, ceftazidime and cefotaxime) were subjected to PCR for the prevalence of ESBL and MBL gene characterization. Out of the 515 isolates, 235 (45.63%) were considered as ESBL positive; 87 (16.89%) were MBL positive and 74 (14.36%) had co-produced both ESBL and MBL. The frequency of TEM-type, SHV-type and AMP-C type ESBLs were 45.10, 26.0, and 28.93%, respectively. Among the MBLs, the frequency of distribution of NDM-1, IMP-1 and VIM-1 was 24.13, 28.73 and 47.12%, respectively. The rate of susceptibility of ESBL producing *P. aeruginosa* towards various antibacterial agents were as follows: piperacillin+tazobactam (84.3%), doripenem (83.8%), ceftriaxone plus ethylenediamminetetraacetic acid plus sulbactam; Elores (74.1%), imipenem (66.5%), meropenem (54.7%), ceftazidime (44.8%) and cefepime (28.5%). Isolates harboring MBL and ESBL+MBL genes were resistant to almost all antibiotics except Elores (97.3 and 95.1% susceptibility) and doripenem (11.3 and 19.5% susceptibility). From the above results, it can be concluded that Elores was highly potent against MBL producing *P. aeruginosa*. However, suceptibility of Elores to ESBL producing *P. aeruginosa* was comparable to piperacillin plus tazobactam and doripenem.

Keywords: Antibiotic adjuvant entity; Clinical isolates; *Pseudomonas aeruginosa*; Extended-spectrum β-lactamases (ESBLS); Metallo-β-lactamases (MBLS)

Introduction

Pseudomonas aerugionosa is one of the most troublesome pathogens implicated in variety of infections including septicemia [1], chronic suppurative otitis media [2], lower respiratory tract infections [3], cystic fibrosis [4] and pneumonia [5]. Aminoglycosides, fluroquinolones, cephalosporins and carbapenems have been used for the treatment of infections caused by *P. aerugionosa* [1,5,6]. However, a decreased susceptibility rate of *P. aeruginosa* to β-lactams, carbapenems, quinolones and aminoglycosides has been reported in various countries [2,7]. Among the cephalosporins, ceftazidime is the most frequently prescribed drug in treating pseudomonal infections because of its unique anti-pseudomonal activity. However, resistance to ceftazidime is increasing alarmingly in recent years [3,8].

Resistance to multiple drugs is usually the result of combination of different mechanism in a single isolate [9]. There are variety of mechanisms involved in the resistance of *P. aeruginosa,* among them over expression of efflux pump [10], acquisition of Extended-Spectrum β-Lactamases (ESBLs) and Metallo-β-Lactamases (MBLs) [11]; target site or outer membrane modification [9] are predominant. Production of multiple-β-lactamases by *P. aeruginosa* has led tremendous therapeutic consequences and posed clinical challenges [8]. ESBLs mediate resistance to extended spectrum cephalosporins such as cefotaxime, ceftriaxone and ceftazidime [12]. The carbapenems and β-lactam and β-lactamase inhibitor combination such as piperacillin plus tazobactam are the drugs active against ESBL producing *P. aeruginosa*. However, resistance to these drugs has also been increasing worldwide [13-15]. The production of MBLs, increased expression of efflux pump, reduced level of drug accumulation are the main factors involved in carbapenem resistance to *P. aeruginos*a [13,16,17]. In India, the prevalence of MBLs ranges from 7.5% to 71% [18]. It has been demonstrated that MBLs require divalent cations, usually zinc, as metal co-factor for their enzymatic activity and no therapeutic option is known to be available to control MBLs [19].

The increasing rate of the antibiotic resistance to commonly used antibacterial agents for the treatment of *P. aeruginosa* infections and its impact on treatment failure encouraged us to find out new strategy by which increasing failure rate of antibiotics in treatment can be controlled. As far as authors know, this may be one of the few studies which have included antibiotic resistance analysis in *P. aeruginosa* co-produced both ESBLs and MBLs.

In view of the grave consequences of drug resistant, we compared susceptibility of a new Antibiotic Adjuvant Entity (AAE) which is a combination of a non-antibiotic adjuvant ethylenediamine tetraacetic acid disodium (disodium edetate) along with β-lactam and β-lactamase inhibitor herein after termed as Elores. The aim of the present study was to investigate the diversity and frequency of both ESBL and MBL production among *P. aeruginosa* clinical isolates obtained from different clinical specimens and to evaluate the drug susceptibility of *P. aeruginosa* isolates.

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Materials and Methods

Bacterial isolates and their identification

A total of 515 clinical isolates of *P. aeruginosa* were prospectively collected from blood (n=241), sputum (121), pus (153) from different centres of India including Delhi, Kolkata, Hyderabad, Bangalore, Aligarh and Chandigarh (name of centers is not disclosed due to confidentiality agreement). Each clinical isolate was collected from different individual. The study was conducted between the period of November 2011 to October 2012. The identification of all isolates was performed using conventional methods [20].

Antibiotics

The following antibiotics were used in this study: ceftriaxone plus EDTA plus sulbactam; Elores (30:10:15 µg), piperacillin plus tazobactam (100:10 µg), cefepime (30 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg) and doripenem (10 µg). All of the discs were obtained from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed by the disc diffusion method according to the procedure of Clinical Laboratory Standard Institute guidelines (CLSI, 2010). *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Stenotrophomonas maltophilia* ATCC 13636 were used as the reference strain throughout study.

Screening of isolates for ESBL and MBL production

Screening of *P. aeruginosa* isolates for ESBLs and MBLs production was performed according to the procedures as recommended by the CLSI [21], using indicator cephalosporins, ceftriaxone (30 µg), ceftazidime (30 µg) and cefotaxime (30 µg). The respective zone size was interpreted according to the recommendations of CLSI [21]. Isolates exhibiting zone size ≤ 25 with ceftriaxone, ≤ 22 for ceftazidime and ≤ 27 with cefotaxime were considered possible ESBLs producer. Similarly, phenotypic detection of MBLs among the suspected ESBLs producer clinical isolates of *P. aeruginosa* was carried out using imipenem (10 µg) and imipenem (10 µg)+EDTA (750 µg) discs as described earlier [22]. Screening of AmpC β-lactamase was done according to the method described elsewhere [23].

Genotypic detection of ESBL and MBL genes

A PCR assay was performed to detect ESBL and MBL encoding genes using the specific primers, namely, TEM-1, TEM-2, TEM-50, SHV-1, SHV-10, AMP-C, NDM-1, VIM-1 and IMP-1 [7,24-30]. All of the respective primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India. For PCR amplifications, about 200 pg of DNA was added to 20 µl mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 1.5 unit of Taq polymerase (Bangalore Genei) in 1x PCR buffer. Amplification was performed in an eppendorf thermal cycler (Germany). The amplified products were separated in 1.5% agarose gel containing 2.5 µl of 10 mg/ml ethidium bromide. The gel was run at 70 volt for 1 h. The gel images were taken under ultraviolet light using gel documentation system (Bio-Rad, USA). A 100 bp ladder was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were visualized using a gel documentation system (Bio-Rad, USA).

DNA isolation

DNA isolation from the clinical isolates was conducted using the alkaline lysis method [31].

Results

Identification and screening of *P. aeruginosa*

All of the clinical isolates were confirmed to be *P. aeruginosa*. The source of all clinical isolates is shown in table 1. Of the 515 clinical isolates of *P. aeruginosa* tested, 235 (45.63%) were identified as ESBL positive. Maximum ESBL production was found in blood (47.71%) followed by pus (44.44%) and sputum (42.97%). Approximately, 87 (16.89%) isolates were MBL positive with dominant in blood (17.42%) followed by sputum (17.35%) and pus (15.68%). In addition, 74 (14.36%) of the isolates had co-produced both ESBL and MBL which were predominantly present in sputum (19.0%) followed by blood (13.27%) and pus (12.41%). The remaining 119 (23.10%) were non-ESBL. The non-ESBLs were predominant in pus (27.45%) followed by blood (21.57%) and sputum (20.66%).

Diversity of ESBLs and MBLs

Results obtained in the present study showed that TEM-type ESBLs (*bla*$_{TEM-1}$, *bla*$_{TEM-2}$, *bla*$_{TEM-50}$) were found in approximately 45.10% of the isolates. The prevalence of SHV-type and AMP-C type ESBLs appeared to be 26.0 and 28.93%, respectively. Among the MBLs, the frequency of distribution of NDM-1, IMP-1 and VIM-1 was 24.13, 28.734 and 47.12%, respectively. The detailed distribution of ESBLs+MBLs is illustrated in table 2.

Antimicrobial susceptibilities of clinical isolates

Results of the antimicrobial susceptibility testing on 515 *P. aeruginosa* isolates collected from November 2011 to October 2012 to various antimicrobial agents are presented in table 3. Among the *P. aeruginosa* collected, all of the antibacterial agents were most active with 100% susceptibility to non-ESBL isolates. Susceptibility among the isolates positive with ESBL for piperacillin+tazobactam was 84.3% followed by doripenem (83.8%), Elores (74.1%), imipenem (66.5%), meropenem (54.7%) ceftazidime (44.8%) and cefepime (28.5%). The isolates obtained from Kolkata region were found to be resistant to both imipenem and meropenem, where as isolates from Hyderabad and Bangalore region were more resistant to ceftazidime and cefepime, and isolates received from Delhi, Aligarh and Chandigarh were more resistant to piperacillin plus tazobactam. This varied trend in resistance may be due to overuse of certain drugs regionally. The most active antibacterial agent against MBL producing isolates was Elores with 97.3% susceptibility, while other comparator antibacterial agents except doripenem were almost 100% resistant to MBL producing isolates, indicating that the Elores and to some extent doripenem was active against MBL producing isolates. Similarly, Elores was the only antibacterial agent found to be active against ESBL+MBL producing isolates with 95.1% susceptibility.

Table 1: Source of clinical isolates.

Source	No. of isolates	ESBL	MBL	ESBL+MBL
Blood	241	115	42	32
Sputum	121	52	21	23
Pus	153	68	24	19

Table 2: Frequency of ESBLs and MBLs genes among *P. aeruginosa* clinical isolates.

Types of resistant determinants (no. of isolates)	Prevalence of genes	No. of isolates	Distribution (%)
ESBL (235)	TEM-1	51	45.10
	TEM-2	31	
	TEM-50	24	
	SHV-1	33	26.0
	SHV-10	28	
	AMP-C	68	28.93
MBL (87)	NDM-1	21	24.13
	IMP-1	25	28.73
	VIM-1	41	47.12
ESBL+MBL (74)	TEM-1+NDM-1	16	21.62
	TEM-2+IMP	13	17.56
	AMP-C+VIM	11	14.86
	AMP-C+NDM-1	16	21.62
	TEM-2+NDM-1	10	13.51
	SHV-1+NDM-1	8	10.81

Discussion

The emergence of multi-drug resistant *P. aeruginosa* has complicated treatment decision and may lead to treatment failures. In the present investigation, a total of 515 clinical isolates of *P. aerugionsa* collected from various clinical specimens and were subjected to screening of ESBLs and MBLs. Based on the results obtained during this study, 45.63% of the isolates were found to be ESBL positive, which contrast to an earlier study showing 20.27% of ESBL production in *P. aeruginosa* [32]. Furthermore, our results showed 16.89% of isolates were MBL producers. Several studies have documented the prevalence of MBLs among *P. aeruginosa* varying from 7.5 to 20.8% [14,33]. Interestingly, in this investigation, approximately 14.36% of the isolates had co-produced both ESBL and MBL, which is contrast to previous study who demonstrated that none of the *P. aeruginosa* isolates co-produced ESBL and MBL [19]. Our data clearly show that the prevalence of ESBL, MBL and ESBL+MBL in *P. aeruginosa* has been increasing which leads to a therapeutic challenge.

When comparison of the antimicrobial susceptibility levels of 4 groups of *P. aeruginosa* isolates were performed, it was found that more than 74 to 97% isolates positive with ESBL, MBL and ESBL+MBL were susceptible to Elores. The enhanced susceptibility of ceftriaxone plus disodium edetate plus sulbactam (Elores) against *P. aeruginosa* is likely to be associated with synergistic activity of ceftriaxone plus sulbactam plus disodium edetate. The authors hypothesized that disodium edetate present in Elores enhanced permeability of ceftriaxone and sulbactam and thereby enhancing activity against ESBL microbes synergistically. Disodium edetate would also chelate the divalent ions required for the activity of MBLs thus de-activating the MBLs which in turn enhanced susceptibility of Elores towards MBLs producing organisms. Although, in the present investigation, we have not studied the expression of efflux pump of *P. aeruginosa,* in a recent publication we have demonstrated that presence of disodium edetate down regulates the expression MexAB-OprM efflux pump of *P. aeruginosa* as well as altering the outer membrane permeability which in turn increased penetration of drugs inside the bacterial cells. This hypothesis is supported by our recently published data [16,34,35].

We observed that only 84.3% isolates positive with ESBL were found to be susceptible and 3.5% were resistant to piperacillin plus tazobactam. Our finding differ from those of [36,37], who observed only 6.06% of *P. aeruginosa* were susceptible to piperacillin-tazobactam. The isolates positive with MBL and ESBL+MBL were 100 and 85.3% resistant to piperacillin plus tazobactam. Contrary to this finding, it was observed that *P. aeruginosa* strains producing MBLs were 81% sensitive to piperacillin+tazobactam [38].

Our results revealed that cefepime was found resistant in 100 and 91.8% of the MBL and ESBL+MBL positive isolates whereas the isolates positive with ESBL showed 65.2% resistant. Resistant could be due to presence of ESBL and MBLs.

In the present investigation, 44.3, 100, 88.5% of the isolates positive with ESBL, MBL and ESBL+MBL, respectively, showed resistance against ceftazidime. In earlier studies, ceftazidime resistance was 28% and 39.6%, respectively in *P. aeruginosa* [39,40]. This high resistance is mainly due to production of ESBL and MBL. Several authors have described the increased resistance in *P. aeruginosa* is due to ESBL and MBL production and over expression of efflux pump [39,41].

The carbapenem and piperacillin plus tazobactam drugs are thought to be the most active drugs against *P. aeruginosa* [42]. In our study, among the penem drugs, doripenem was found to be more active with 83.8% susceptibility against ESBL positive *P. aeruginosa,* whereas it was less active against MBL and ESBL+MBL with 11.3 and 19.5% susceptibility. Furthermore, 66.5% of ESBL positive isolates were found to be sensitive while 17.7% showed intermediate response and 15.8% were found to be resistant to imipenem. Similarly for meropenem, 54.6% of ESBL positive isolates were found to be sensitive whereas 31.7% were intermediate response and 13.6% were appeared to be resistant. Meropenem and imipenem were found to be 100% resistant to MBL positive isolates. The isolates positive with ESBL+MBL showed 90.2 and 88.5% resistance to imipenem and meropenem, respectively.

Table 3: Antibiotic susceptibility of the clinical isolates of *P. aeruginosa.*

Antimicrobial agent	Percentage (%) of isolates											
	Non-ESBL			ESBL			MBL			ESBL+MBL		
	S	I	R	S	I	R	S	I	R	S	I	R
CSE1034	100	0	0	74.1	22.7	3.1	97.3	0	0	95.1	1.6	3.3
Piperacillin+tazobactam	100	0	0	84.3	12.2	3.5	0	0	100	0	14.7	85.3
Cefepime	100	0	0	28.5	6.3	65.1	0	0	100	0	8.2	91.8
Ceftazidime	100	0	0	44.8	10.8	44.3	0	0	100	0	11.5	88.5
Imipenem	100	0	0	66.5	17.7	15.8	0	0	100	0	9.8	90.2
Meropenem	100	0	0	54.7	31.7	13.6	0	0	100	0	11.5	88.5
Doripenem	100	0	0	83.8	12.8	3.4	11.3	86.6	2.1	19.5	77.7	2.8

Here, S=susceptible; I: intermediate; R=resistant.

It has been reported that resistance of imipenem and meropenem in *P. aeruginosa* due to the presence of nonenzymatic mechanism of carbapenem resistance such as porin loss and/or overexpression of efflux pumps [39]. Our data clearly showed that at least in North India *P. aeruginosa* possess high levels of resistance to ceftazidime, and piperacillin plus tazobactam and could become serious health problems.

Our study clearly demonstrate that the frequency of ESBL and MBL mediated resistance among the *P. aeruginosa* is increasing and the drugs commonly used for the treatment of *P. aeruginosa* infections are getting resistant. Results obtained in the present investigation demonstrate the potent *in-vitro* activity of Elores against MBLs producing *P. aeruginosa*. However, penems and Piperacillin plus tazobactam exhibited *in-vitro* activity against only ESBLs producing *P. aeruginosa*. Hence, in case of infection with MBLs producing *P. aeruginosa*, ceftriaxone plus disodium edetate plus sulbactam (Elores) can be of drug of choice for the treatment.

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