Role of inducers in detection of bla_{PDC}-mediated oxyimino-cephalosporin resistance in *Pseudomonas aeruginosa*

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Background & objectives: *Pseudomonas aeruginosa* possessing chromosomally inducible bla_{PDC} along with other intrinsic mechanism causes infection with high mortality rate. It is difficult to detect inducible AmpC enzymes in this organism and is usually overlooked by routine testing that may lead to therapeutic failure. Therefore, three different inducers were evaluated in the present study to assess their ability of induction of bla_{PDC} in *P. aeruginosa*.

Methods: A total of 189 consecutive *Pseudomonas* isolates recovered from different clinical specimens (November 2011-April 2013) were selected for the study. Isolates were screened with cefoxitin for AmpC β-lactamases and confirmed by modified three-dimensional extract test (M3DET). Inductions were checked using three inducers, namely, clavulanic acid, cefoxitin and imipenem along with ceftazidime. Molecular screening of AmpC β-lactamase genes was performed by PCR assay. Antimicrobial susceptibility and minimum inhibitory concentrations (MICs) were determined, and repetitive extragenic palindromic-PCR of all bla_{PDC} harbouring isolates was performed.

Results: Inducible phenotype was observed in 42 (24.3%) of 97 (56%) isolates confirmed by M3DET. Among these, 22 isolates harboured chromosomal bla_{PDC} gene, and cocarriage of both chromosomal and plasmid-mediated bla_{AmpC} genes was observed in seven isolates. Cefoxitin-ceftazidime-based test gave good sensitivity and specificity for detecting inducible AmpC enzymes. Isolates harbouring bla_{PDC} showed high MIC against all tested cephalosporins and monobactam. DNA fingerprinting of these isolates showed 22 different clones of *P. aeruginosa*.

Interpretation & conclusions: *P. aeruginosa* harbouring inducible (chromosomal) and plasmid-mediated AmpC β-lactamase is a matter of concern as it may limit therapeutic option. Using cefoxitin-ceftazidime-based test is simple and may be used for detecting inducible AmpC β-lactamase amongst *P. aeruginosa*.

Key words AmpC β-lactamase - bla_{PDC} - *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the most common nosocomial pathogens that causes infection with high mortality rate[1]. Antipseudomonal chemotherapy for these organisms is of great concern since these organisms possess chromosomally inducible *Pseudomonas*-derived cephalosporinases (PDC) with broad-spectrum activity along with other intrinsic mechanisms. Further, it may also possess other inducible plasmid-mediated AmpC β-lactamases such as ACT-1, DHA-1, DHA-2, DHA-23 and CMY-13 that could limit therapeutic option[2-4]. Induction of AmpC β-lactamases takes place mainly in response to several β-lactam and non-β-lactam antibiotics such as penicillin group of drugs (ampicillin and amoxicillin) and cephalosporins group of drugs (cefazolin, cephalothin and cefoxitin) which act as strong inducers and good substrates for AmpC β-lactamase. Carbapenem group of drug (imipenem) also acts as strong inducers but is much more stable for hydrolysis. Cefotaxime, ceftriaxone, ceftazidime, cefepime, cefuroxime, piperacillin and aztreonam are weak inducers and weak substrates but can be hydrolyzed if enough enzymes are synthesized[5,6]. Other non-β-lactam antibiotics such as clavulanic acid also act as an inducer[7]. Currently, there is no Clinical and Laboratory Standards Institute (CLSI) guideline for phenotypic screening or confirmatory test for inducible AmpC β-lactamase producing bacteria. Based on inducing capabilities of imipenem on AmpC β-lactamase, ceftazidime-imipenem antagonism test (CIAT) was developed which showed good efficiency in detecting inducible AmpC β-lactamase amongst *Enterobacteriaceae* familly[8]. Since *P. aeruginosa* involves various intrinsic resistance mechanisms other than harbouring β-lactamases; it is difficult to detect accurate inducible AmpC β-lactamase in this organism[9,10]. Therefore, three different inducers were evaluated in this study to assess their ability of induction of bla_{AmpC} in *P. aeruginosa*.

Material & Methods

A total number of 189 consecutive, non-duplicates, *Pseudomonas* isolates were selected for this study. These isolates were collected from different clinical specimens (urine, pus, stool, ear swab, throat swab, oral swab, sputum, ascitic fluid, drain tip, blood, conjunctival scraping, urethral discharge and ear discharge) spanning a period of 18 months (November 2011-April 2013) from different Wards/OPD of Silchar Medical College and Hospital, Silchar, Assam, India. The isolates were identified by cultural characteristics, pigment production and 16S rDNA sequencing.

Screening of ampC β-lactamase by cefoxitin and modified three-dimensional extract test (M3DET): Preliminary screening of AmpC β-lactamase was carried out on Mueller-Hinton agar (MHA) (HiMedia, Mumbai) plates containing single antibiotic disk, namely cefoxitin (30 μg) (HiMedia). Isolates with inhibition zones of <18 mm to cefoxitin were considered as screen positives isolates[11]. The suspected AmpC β-lactamase producers were further confirmed by M3DET[12]. *Escherichia coli* ATCC 25922 and *Enterobacter cloacae* P99 were used as a negative and positive control, respectively.

Detection of inducible ampC β-lactamase: Induction experiment was performed with slight modification of CIAT[8]. Imipenem (10 μg), cefoxitin (30 μg) and clavulanic acid (30 μg) (HiMedia) were used to check the potentiality to induced AmpC β-lactamase. The discs were placed 20 mm apart (centre-to-centre) from a ceftazidime disk (30 μg) on a MHA plate previously inoculated with a 0.5 McFarland bacterial suspension and incubated for 24 h at 37°C. Antagonism, indicated by a visible reduction in the inhibition zone around the ceftazidime disk adjacent to the imipenem, clavulanic acid or cefoxitin disk, was regarded as positive for inducible AmpC β-lactamase production. Sensitivity and specificity of the tests were evaluated by statistical analysis.

Antimicrobial susceptibility and minimum inhibitory concentrations (MICs): Antimicrobial susceptibility was determined by Kirby–Bauer disc diffusion method[13] on MHA plates. The following antibiotics were used: amikacin (30 μg), gentamicin (10 μg), ciprofloxacin (30 μg), trimethoprim/sulphamethoxazole (1.25/23.75 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg), ceftriaxone (30 μg) and aztreonam (30 μg) (HiMedia). Minimum inhibitory concentrations (MICs) of various antibiotics were determined on MHA plates by agar dilution method according to CLSI guidelines[14] using the following antibiotics: cefotaxime, ceftazidime, ceftriaxone, cefepime, imipenem, meropenem, ertapenem and aztreonam (HiMedia).

Detection of bla_{PDC} by PCR: For amplification and characterization of bla_{PDC} genes, forward 5'-ATGCAGCCAACGACAAAGG-3' and reverse primers 5'-CGCCCTCGCGAGCGCGCTTC-3' were used[2]. PCR amplification was performed using 30 μl of total reaction volume. Reactions were run under the

following conditions: initial denaturation at 95°C for two minutes, 32 cycles of 95°C for 20 sec, 58°C for 40 sec, 72°C for 1.2 min and final extension at 72°C for 7 min. The PCR product was run on 1 per cent agarose gel and visualized in Gel Doc EZ imager (Bio-Rad, USA).

Multiplex PCR assay for detection of plasmid-mediated AmpC β-lactamase: Multiplex PCR was performed on all the AmpC β-lactamase positive isolates targeting all the AmpC β-lactamase gene families, namely, CIT, DHA, ACC, FOX and EBC[15]. PCR amplification was performed using 30 µl of total reaction volume. Reactions were run under the following conditions: initial denaturation at 95°C for two minutes, 34 cycles of 95°C for 15 sec, 51°C for one minute, 72°C for one minute and final extension at 72°C for seven minutes. The PCR product was run on 1 per cent agarose gel and visualized in Gel Doc EZ imager (Bio-Rad, USA).
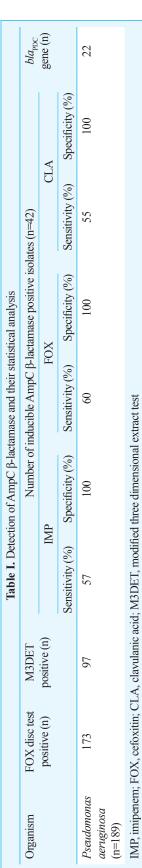
DNA fingerprinting by repetitive extragenic palindromic (REP) PCR: Typing of all bla_{PDC} producing isolates was done by REP- PCR as described previously[16].
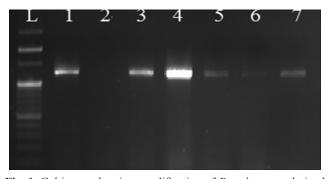
Statistical analysis: Sensitivity and specificity of the phenotypic screening tests for the detection of AmpC β - lactamase was done when compared with three different inducers *i.e.*, imipenem, cefoxitin and clavulanic acid. Statistical analysis was performed taking M3DET as gold standard.
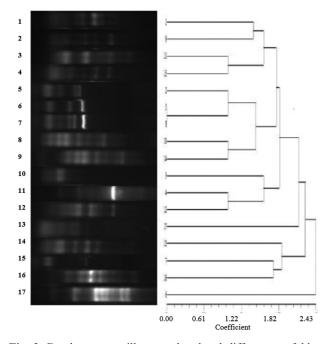
Results

Of the 189 isolates, 173 (91.5%) were resistant to cefoxitin. On performing M3DET, 97 (56%) isolates were found to show AmpC β-lactamase activity. However, inducible AmpC β-lactamase was detected only in 42 (24.3%) isolates. A higher number of induction was observed against cefoxitin (n=31) followed by imipenem (n=25) and clavulanic acid (n=17) (Table I). In contrast, seven isolates showed induction against both imipenem and cefoxitin, one isolate for both imipenem and clavulanic acid, four isolates for both cefoxitin and clavulanic acid and seven isolates showed induction against all the three inducers.

Genotypic detection of chromosomal AmpC β-lactamase showed that 22 isolates harboured bla_{PDC} gene (1243bp) (Fig. 1). Of the 22 chromosomal harbouring β-lactamase isolates, 19 showed

Table I. Detection of AmpC β-lactamase and their statistical analysis

Organism	FOX disc test positive (n)	M3DET positive (n)	Number of inducible AmpC β-lactamase positive isolates (n=42)						bla_{PDC} gene (n)
			IMP		FOX		CLA		
			Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	
Pseudomonas aeruginosa (n=189)	173	97	57	100	60	100	55	100	22

IMP, imipenem; FOX, cefoxitin; CLA, clavulanic acid; M3DET, modified three dimensional extract test

Fig. 1. Gel image showing amplification of *Pseudomonas*-derived cephalosporinases gene: Lane L, 100 bp DNA ladder; lanes 1, 3, 4, 5, 6, 7, *Pseudomonas*-derived cephalosporinases gene (1243 bp); lane 2, negative control.

Fig. 2. Dendrogram to illustrate the clonal differences of bla_{PDC} producing *Pseudomonas aeruginosa* isolates.

Fig. 3. Antimicrobial susceptibility patterns of AmpC positive isolates against β-lactam and non-β-lactam antibiotics. CRO-ceftriaxone, FEP- cefepime, ATM-aztreonam, IMP-imipenem, MEM-meropenem, AMK-amikacin, GEN-gentamicin, CIP-ciprofloxacin, SXT-co-trimoxazole, TGC- tigecycline

Table II. Minimum inhibitory concentrations (MIC) of isolates harbouring bla_{PDC} gene against different β-lactam drugs

Antimicrobial agents	MIC_{50}	MIC_{90}
Cefotaxime	256	>512
Ceftriaxone	128	512
Ceftazidime	64	>512
Cefepime	32	256
Aztreonam	128	256
Imipenem	<4	8
Meropenem	<4	8
Ertapenem	<4	4

phenotypic inducibility. On performing multiplex PCR, plasmid-mediated bla_{AmpC} genes were detected among 51 isolates and were found to be CIT, DHA and EBC family (20, CIT; 19, DHA and 12, EBC). However, only 14 plasmid-mediated bla_{AmpC} genes were observed among phenotypically inducible AmpC β-lactamase-positive isolates. It was also observed that seven isolates were harbouring both chromosomal and plasmid-mediated bla_{AmpC} genes.

M3DET was used as gold standard for calculating sensitivity and specificity. For imipenem-ceftazidime-based test, sensitivity was found out to be 57 per cent, cefoxitin-ceftazidime based test 60 per cent and clavulani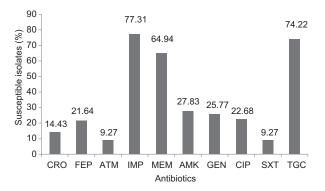c acid-ceftazidime based test 55 per cent. All these tests showed 100 per cent specificity for detecting inducible AmpC β-lactamases (Table I). DNA fingerprinting of all the bla_{PDC} harbouring isolates by repetitive PCRs showed 17 different clones of *P. aeruginosa* (Fig. 2).

Susceptibility results showed that most of the bla_{PDC} harbouring isolates were susceptible to carbapenem group of drugs. Other antibiotics such as ceftriaxone, cefepime, amikacin, gentamicin, ciprofloxacin and co-trimoxazole showed moderate to lower activity (Fig. 3). A high MIC was noticed against all tested cephalosporins and monobactam; however, MIC_{50} of isolates against carbapenems were at the susceptible range (Table II).

Discussion

Organisms producing chromosomally inducible AmpC β-lactamase are usually overlooked by routine testing as these often do not show resistance to third-generation cephalosporins. Clinical use of cephalosporins against these isolates could segregate

resistant mutants that would ultimately result in therapeutic failure. Therefore, current detection methods for screening of inducible AmpC β-lactamase producing organisms are technically demanding. There is also a paucity of data regarding inducible AmpC β-lactamases worldwide. It was observed that 24.3 per cent isolates in the present study harboured these enzymes which corroborated with earlier studies[17,18]. Of the three inducers used in this study, cefoxitin showed higher ability to induce AmpC β-lactamase with sensitivity of 60 per cent followed by imipenem and clavulanic acid. This finding was supported by another study where cefoxitin was established to be a potent inducer of class C β-lactamase in *P. aeruginosa*[19]. Clavulanic acid showed moderate inducing capabilities as reported in earlier studies[7,20].

Induction phenotype observed in *P. aeruginosa* could be due to PDC which has three AmpD homologues to regulate the expression of AmpC β-lactamase[21,22]. In the present study bla_{PDC} gene was screened in *P. aeruginosa* isolates that were phenotypically confirmed for AmpC β-lactamase activity and 23 per cent isolates showed bla_{PDC} gene which was supported by earlier study[23]. It was observed that 19 isolates harbouring bla_{PDC} showed inducible AmpC phenotype. These isolates showed resistant profile against third- and fourth-generation cephalosporins and monobactams but most of these showed susceptible profile against carbapenem antibiotics. Ten variants of bla_{PDC} (PDC 1-10) gene have been reported worldwide which have reduced susceptibility for third- and fourth-generation cephalosporin and carbapenem[2].

The discovery of inducible chromosome-mediated AmpC β-lactamase requires re-evaluation of the treatment option available for patients infected with pathogens expressing this resistance mechanism. Presence of additional plasmid-mediated AmpC β-lactamase showing expanded spectrum cephalosporin-resistant phenotype would further complicate the antibiotic policy and therapeutic options in nosocomial infections caused by these isolates. Use of cefoxitin along with ceftazidime is a simple method and may be used for routine detection of chromosomally inducible AmpC β-lactamase amongst *P. aeruginosa*.

Acknowledgment

Conflicts of Interest: None.

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