Role of inducers in detection of bla<sub>PDC</sub>-mediated oxyiminocephalosporin resistance in Pseudomonas aeruginosa

Birson Ingti<sup>1</sup>, Deepika B. Krishnatreya<sup>1</sup>, Anand Prakash Maurya<sup>1</sup>, Debadatta Dhar (Chanda)<sup>2</sup>, Atanu Chakravarty<sup>2</sup> & Amitabha Bhattacharjee<sup>1</sup>

<sup>1</sup>Department of Microbiology, Assam University & <sup>2</sup>Department of Microbiology, Silchar Medical College & Hospital, Silchar, India

Received April 25, 2015

**Background & objectives:** Pseudomonas aeruginosa possessing chromosomally inducible bla<sub>PDC</sub> 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<sub>PDC</sub> 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<sub>PDC</sub> 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<sub>PDC</sub> gene, and cocarriage of both chromosomal and plasmid-mediated bla<sub>AmpC</sub> genes was observed in seven isolates. Cefoxitin-ceftazidime-based test gave good sensitivity and specificity for detecting inducible AmpC enzymes. Isolates harbouring bla<sub>PDC</sub> 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<sub>PDC</sub> - Pseudomonas aeruginosa
Pseudomonas aeruginosa is one of the most common nosocomial pathogens that causes infection with high mortality rate. 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. 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, cephalexin 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, ceftazidime, cefepime, cefuroxime, piperacillin and aztreonam are weak inducers and weak substrates but can be hydrolyzed if enough enzymes are synthesized. Other non-β-lactam antibiotics such as clavulanic acid also act as an inducer. 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 family. 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. Therefore, three different inducers were evaluated in this study to assess their ability of induction of blaAmpC 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 (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. The suspected AmpC β-lactamase producers were further confirmed by M3DET. 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. 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 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), cefotaxime (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 using the following antibiotics: cefotaxime, ceftazidime, ceftriaxone, cefepime, imipenem, meropenem, ertapenem and aztreonam (HiMedia).

Detection of blaPDC by PCR: For amplification and characterization of blaPDC genes, forward 5′-ATGCAGCCAACGACAAAGG-3′ and reverse primers 5′-CGCCCTCGGAGCGCGGTTC-3′ were used. 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. 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 blaPDC producing isolates was done by REP-PCR as described previously.

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 blaPDC gene (1243bp) (Fig. 1). Of the 22 chromosomal harbouring β-lactamase isolates, 19 showed
showed 100 per cent specificity for detecting inducible AmpC β-lactamases (Table I). DNA fingerprinting of all the blaPDC-harbouring isolates by repetitive PCRs showed 17 different clones of P. aeruginosa (Fig. 2).

Discussion

Organisms producing chromosomally inducible AmpC β-lactamases 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

**Table II.** Minimum inhibitory concentrations (MIC) of isolates harbouring blaPDC gene against different β-lactam drugs

| Antimicrobial agents | MIC<sub>50</sub> | MIC<sub>90</sub> |
|----------------------|-----------------|-----------------|
| Cefotaxime           | 256             | >512            |
| Ceftriaxone          | 128             | 512             |
| Ceftazidime          | 64              | >512            |
| Cefepime             | 32              | 256             |
| Aztreonam            | 128             | 256             |
| Imipenem             | <4              | 8               |
| Meropenem            | <4              | 8               |
| Ertapenem            | <4              | 4               |

Susceptibility results showed that most of the blaPDC harbouring isolates were susceptible to carbapenem group of drugs. Other antibiotics such as ceftriaxone, cefepime, amikacin, gentamicin, ciprofloxacin and tobramycin showed moderate to lower activity (Fig. 3). A high MIC was noticed against all tested cephalosporins and monobactam; however, MIC<sub>90</sub> of isolates against carbapenems were at the susceptible range (Table II).
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\textsuperscript{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 \textit{P. aeruginosa}\textsuperscript{19}. Clavulanic acid showed moderate inducing capabilities as reported in earlier studies\textsuperscript{2,20}.

Induction phenotype observed in \textit{P. aeruginosa} could be due to PDC which has three AmpD homologues to regulate the expression of AmpC β-lactamase\textsuperscript{21,22}. In the present study \textit{bla}_{\text{PDC}} gene was screened in \textit{P. aeruginosa} isolates that were phenotypically confirmed for AmpC β-lactamase activity and 23 per cent isolates showed \textit{bla}_{\text{PDC}} gene which was supported by earlier study\textsuperscript{23}. It was observed that 19 isolates harbouring \textit{bla}_{\text{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 \textit{bla}_{\text{PDC}} (PDC 1-10) gene have been reported worldwide which have reduced susceptibility for third- and fourth-generation cephalosporin and carbapenem\textsuperscript{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 \textit{P. aeruginosa}.

Acknowledgment

The authors acknowledge the help of Head, Department of Microbiology, Assam University, for providing infrastructure. Financial support provided by University Grants Commissions UGC-MRP and UGC-RGNF, Government of India and Department of Biotechnology (DBT-NER Twinning Scheme) is acknowledged. Authors thank the Assam University Biotech Hub for providing laboratory facility to complete this work.

Conflicts of Interest: None.

References

1. Vidal F, Mensa J, Almela M, Martinez JA, Marco F, Casals C, \textit{et al.} Epidemiology and outcome of \textit{Pseudomonas aeruginosa} bacteremia, with special emphasis on the influence of antibiotic treatment Analysis of 189 episodes. \textit{Arch Intern Med} 1996; 156 : 2121-6.

2. Rodríguez-Martínez JM, Poirel L, Nordmann P. Extended-spectrum cephalosporinases in \textit{Pseudomonas aeruginosa}. \textit{Antimicrob Agents Chemother} 2009; 53 : 1766-71.

3. Jacoby GA. AmpC beta-lactamases. \textit{Clin Microbiol Rev} 2009; 22 : 161-82.

4. Hsieh WS, Wang NY, Fung JA, Weng LC, Wu HH. Identification of DHA-23, a novel plasmid-mediated and inducible AmpC beta-lactamase from \textit{Enterobacteriaceae} in Northern Taiwan. \textit{Front Microbiol} 2015; 6 : 436.

5. Livermore DM. Beta-lactamases in laboratory and clinical resistance. \textit{Clin Microbiol Rev} 1995; 8 : 557-84.

6. Sanders CC, Sanders WE Jr. Type I beta-lactamases of Gram-negative bacteria: interactions with beta-lactam antibiotics. \textit{J Infect Dis} 1986; 154 : 792-800.

7. Weber DA, Sanders CC. Diverse potential of beta-lactamase inhibitors to induce class I enzymes. \textit{Antimicrob Agents Chemother} 1990; 34 : 156-8.

8. Cantarelli VV, Inamine E, Brodt TC, Secchi C, Cavalcante BC, Pereira Fde S. Utility of the ceftazidime-imipenem antagonism test (CIAT) to detect and confirm the presence of inducible AmpC beta-lactamases among \textit{Enterobacteriaceae}. \textit{Braz J Infect Dis} 2007; 11 : 237-9.

9. Livermore DM. Multiple mechanisms of antimicrobial resistance in \textit{Pseudomonas aeruginosa}: our worst nightmare? \textit{Clin Infect Dis} 2002; 34 : 634-40.

10. Morita Y, Tomida J, Kawamura Y. Responses of \textit{Pseudomonas aeruginosa} to antimicrobials. \textit{Front Microbiol} 2014; 4 : 422.

11. Lorian V. \textit{Antibiotics in laboratory medicine}. 5th ed. Philadelphia: Lippincott Williams and Wilkins; 2005.

12. Coudron PE, Moland ES, Thomson KS. Occurrence and detection of AmpC beta-lactamases among \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, and \textit{Proteus mirabilis} isolates at a veterans medical center. \textit{J Clin Microbiol} 2000; 38 : 1791-6.

13. Biener JJ. Antimicrobial susceptibility testing by Kirby-Bauer disc diffusion method. \textit{Ann Clin Lab Sci} 1973; 3 : 135-40.

14. Clinical and Laboratory Standards Institute. \textit{Performance Standards for Antimicrobial Susceptibility Testing.} 23rd ed. CLSI Document M100-S23. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.

15. Dalleniee C, Da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in \textit{Enterobacteriaceae}. \textit{J Antimicrob Chemother} 2010; 65 : 490-5.

16. Versalovic J, Schneider M, de Bruijn FJ, Lupski JR. Genomic fingerprinting of bacteria using repetitive sequence based polymerase chain reaction. \textit{Methods Mol Cell Biol} 1994; 5 : 25-40.
17. Bhattacharjee A, Anupurba S, Gaur A, Sen MR. Prevalence of inducible AmpC beta-lactamase-producing *Pseudomonas aeruginosa* in a tertiary care hospital in Northern India. *Indian J Med Microbiol* 2008; 26: 89-90.

18. Upadhyay S, Sen MR, Bhattacharjee A, Prakash P, Bajpai RC, Anupurba S. Diagnostic utility of combination of inducer and inhibitor based assay in detection of *Pseudomonas aeruginosa* producing AmpC ß-lactamase. *J Microbiol Methods* 2011; 87: 116-8.

19. Sanders CC, Sanders WE Jr., Goering RV. *In vitro* antagonism of beta-lactam antibiotics by cefoxitin. *Antimicrob Agents Chemother* 1982; 21: 968-75.

20. Stobberingh EE. Induction of chromosomal beta-lactamases by different concentrations of clavulanic acid in combination with ticarcillin. *J Antimicrob Chemother* 1988; 21: 9-16.

21. Juan C, Moyá B, Pérez JL, Oliver A. Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level beta-lactam resistance involves three AmpD homologues. *Antimicrob Agents Chemother* 2006; 50: 1780-7.

22. Caille O, Zincke D, Merighi M, Balasubramanian D, Kumari H, KongKF, *et al.* Structural and functional characterization of *Pseudomonas aeruginosa* global regulator AmpR. *J Bacteriol* 2014; 196: 3890-902.

23. Upadhyay S, Mishra S, Sen MR, Banerjee T, Bhattacharjee A. Co-existence of *Pseudomonas*-derived cephalosporinase among plasmid encoded CMY-2 harbouring isolates of *Pseudomonas aeruginosa* in North India. *Indian J Med Microbiol* 2013; 31: 257-60.

*Reprint requests:* Dr Amitabha Bhattacharjee, Department of Microbiology, Assam University, Silchar 788 014, Assam, India
e-mail: ab0404@gmail.com