RESEARCH ARTICLE

# Expansion of plasmid mediated bla$_{ACT-2}$ among Pseudomonas aeruginosa associated with hospital infection and its transcriptional response under cephalosporin stress

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## ABSTRACT

**Objectives:** Organisms harboring multiple plasmid mediated β-lactamases are major concerns in nosocomial infections. Among these plasmid mediated β-lactamases, ACT (EBC family) is a clinically important enzyme capable of hydrolyzing broad spectrum cephalosporins. Therefore, the present study was undertaken to determine the prevalence of ACT determinant along with other co-existing β-lactamase genes in *P. aeruginosa* strains.

**Methods:** A total of 176 Pseudomonas isolates were phenotypically screened for the presence of AmpC β-lactamase by M3DET Method followed by Molecular detection using PCR assay. Transcriptional evaluation of *bla*$_{ACT-2}$ gene was analyzed by RT-PCR and its transferability was performed by transformation and conjugation.

**Results:** Present study demonstrates the presence of ACT-2 allele among 12 strains of *P. aeruginosa*. Co-existence of other β-lactamase genes were encountered among ACT-2 harboring strains which includes CTX-M (n=2), SHV (n=3), TEM (n=2), VEB (n=2), OXA-10 (n=1), CIT (n=2) and DHA (n=3). Fingerprinting by REP PCR revealed the isolates harboring ACT-2 to be distinct and these isolates showed high resistance to expanded-spectrum cephalosporins and even to carbapenem group of drugs. This ACT-2 allele was encoded in the plasmid (L/M, FIA, FIB Inc. Group) and conjugatively transferable. Transcriptional analysis revealed a significant increase in ACT-2 expression (483 fold) when induced by ceftriaxone at 4 µg/ml followed by ceftazidime at 8 µg/ml (31 fold) and cefotaxime 4 µg/ml (8 fold).

**Conclusion:** In this study detection of ACT-2 plasmid mediated AmpC β-lactamase along with other β-lactamase genes in clinical isolates of *P. aeruginosa* represents a serious therapeutic challenge. Therefore, revision in antimicrobial policy is required for effective treatment of patients infected with pathogen expressing this mechanism. *J Microbiol Infect Dis 2017; 7(2): 75-82*

**Keywords:** *Plasmid mediated, ACT-2 β-lactamase, P. aeruginosa*

## INTRODUCTION

*Pseudomonas aeruginosa,* one of the major causing agent of nosocomial infection is known to exhibit multiple resistance mechanisms and capable of conferring resistance to a range of antibiotics, leaving fewer treatment options for infection caused by this organism. This organism other than having an efflux pump and porin innate genes, it also possesses chromosomally inducible AmpC β-lactamase which is regulated by regulatory genes (AmpG, AmpR and three AmpD homologues (AmpD1, AmpDh2 and AmpDh3) [1-3]. The upregulation of this enzyme mainly depends on the presence of an external stimulus (such as cefoxitin and imipenem) or when there are mutations in the regulatory elements of AmpC leading to a stable, hyperproduction of AmpC β-lactamase [4,5]. *Pseudomonas spp.* is also known to harbor several plasmid mediated β-lactamase genes which include Ambler class A enzymes

(PER, SHV, KPC and GES), class D enzymes (OXA-18,OXA-45), metalloenzymes (IMP, VIM, NDM-1) and class C enzymes (DHA, ACT, MIR, FOX, MOX, ACC, CMY). It has been believed that class C (AmpC) genes carried on plasmids were originated from chromosomal AmpC genes of *Morganilla morganii, Enterobacter cloacae, Pseudomonas aeruginosa, Hafnia alvie* or *Citrobacter freundii* [2,4,6-8].

Plasmid-encoded ACT (EBC family) related to the chromosomal AmpC gene of Enterobacter spp. are clinically important enzymes that can hydrolyse penicillin, cephamycin and even to carbapenem when combining with the loss of a major outer membrane protein [9-11]. Until now, a total of 37 ACT allele types has been reported some of which are inducible [12]. Earlier studies show that these ACT allele types were repeatedly described from *E.coli* and *Klebsiella pneumoniae* [9-14]. Meanwhile, the frequency of this determinant in non-fermenter organisms especially *Pseudomonas aeruginosa* has remained unclear. Therefore, active surveillance and early detection of these β-lactamases are mandatory in order to prevent further spread.

Hence, we undertook this study to investigate the presence of ACT in *P. aeruginosa* and to look for the coexistence of other β-lactamase genes. This study also investigates the expression of an ACT-2 allele in response to different gradients of cephalosporin stress.

## METHODS

### Clinical isolates

A total of 176 consecutive, non-duplicate *Pseudomonas* isolates were collected from different clinical specimens obtained from different Wards/OPD of Silchar Medical College and Hospital, India. The present study was undertaken for a period of 10 months from February to November 2013. The isolates were identified by cultural characteristics, biochemical reactions, and 16S rDNA sequencing.

### Phenotypic detection of AmpC β-lactamases

Phenotypically, screening of AmpC β-lactamases was carried out on Mueller Hinton Agar plates containing cefoxitin disk (30 µg) (Hi Media, Mumbai). Isolates with inhibition zones of less than 18 mm, were considered as screen positive isolates [15]. The suspected AmpC β-

lactamase producers were further confirmed by Modified Three Dimensional Extract Test (M3DET) [16]. *Escherichia coli* ATCC 25922 and *Enterobacter cloacae* P99 were used as negative and positive control respectively.

### Detection of Inducible AmpC β-lactamase

Inducible AmpC β-lactamase was check using three inducers cefoxitin, imipenem and clavulanic acid (10 µg) (Hi Media, Mumbai). The disks were placed 20 mm apart (center-to-center) from a ceftazidime disk (30 µg) (Hi Media, Mumbai) on a Mueller Hinton agar 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 was regarded as positive for inducible AmpC β-lactamase production. *Escherichia coli* ATCC 25922 and *Enterobacter cloacae* P99 were used as negative and positive control respectively.

### Antimicrobial Susceptibility testing

Antimicrobial susceptibility was determined by Kirby Bauer disk diffusion method on Mueller Hinton Agar plates. Following antibiotics were used: amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (30 µg), trimithoprim/ sulphamethoxazole (1.25/23.75µg), tigecycline (15 µg) (Hi Media, Mumbai). MIC's of various antibiotics were also determined on Mueller Hinton agar plates by agar dilution method and the results were interpreted as per CLSI guidelines [17]. Following antibiotics were used for MIC study: cefotaxime, cefotaxime/ tazobactam, cefotaxime/sulbactam, ceftazidime, ceftriaxone, cefepime, imipenem, meropenem, ertapenem, and aztreonam. (Hi-Media, Mumbai, India).

### Molecular screening of AmpC resistance determinants

Initial PCR was performed targeting EBC alleles by using a pair of primers, forward primer 5'-CGGTAAAGCCGATGTTGCG-3' and reverse primer 5'-AGCCTAACCCCTGATACA-3' [18]. Isolates were further investigated for the presence of other AmpC gene families, namely: CIT, ACC, FOX and DHA [18].PCR amplification was performed using 30 µl of total reaction volume. Reactions were run under the following conditions: initial denaturation at 95°C for 2 min,

34 cycles of 95 °C for 15 sec, 51 °C for 1 min, 72°C for 1 min and a final extension at 72 °C for 7 min.

Molecular characterization of ESBL and carbapenemase genes by Multiplex PCR:

For amplification and characterization of ESBL genes, a set of five primers was used, namely: TEM, CTX-M, SHV, OXA-2, and PER [6].Reactions were run under the following conditions: initial denaturation at 94 $^0$C for 5 min, 33 cycles of 94 $^0$C for 35 sec., 51 $^0$C for 1 min, 72 $^0$C for 1 min and a final extension at 72°C for 7 min.

For amplification and characterization of carbapenemase genes, a set of six primers were used, namely: KPC, IMI/NMC, SME, VIM, IMP, and NDM [19-23]. Reactions were run as described previously.

**Plasmid preparation**

The bacterial isolates were being cultured in Luria-Bertani broth (LB broth) containing 0.25 µg/ml of cefotaxime. Cultures were incubated on shaker incubator overnight at 37°C, 160 rpm. Plasmids were purified by QIA prep Spin Miniprep Kit (QIAGEN, Germany).

**Transferability of the bla$_{ACT}$ gene by transformation and conjugation**

The transformation was carried out using *Escherichia coli* DH5α as the recipient. Transformants were selected on cefotaxime (0.5 µg/ml) containing LB Agar plates. The experiment was performed by heat shock method [24].

Conjugation experiment was carried out between donor (transformants harboring ACT-2) and a recipient (streptomycin resistant *Escherichia coli* strain B (Genei, Banglore). Overnight cultures of the bacteria were diluted in Luria-Bertani broth (Hi-Media, Mumbai, India) and were grown at 37 °C till the O.D. of the recipient and donor culture reached 0.8-0.9 at A$_{600}$. Donor and recipient cells were mixed at 1:5 donor-to-recipient ratios and transconjugants were selected on L.B agar plates supplemented with cefotaxime (0.5 µg/ml) and streptomycin (800 µg/ml).

**Plasmid incompatibiltiy typing**

For detection of incompatibility group type of plasmid, PCR-based replicon typing was carried out, targeting 18 different replicon types, to perform 5 multiplex and 3 simplex PCRs to amplify the FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons [25].

**Transcriptional expression analysis of bla$_{ACT}$ by RT-PCR**

The expression of *bla*$_{ACT-2}$ genes was studied in response to cefoxitin, cefotaxime, ceftriaxone, and ceftazidime stress at different concentrations (2, 4, 8 µg/ml). Isolates grown in Luria-Bertani broth (Hi-media, Mumbai, India) for 16 hours without any antibiotic pressure was used as a control. A total RNA was extracted using the Qiagen RNease Mini Kit (Qiagen, Germany), immediately reverse transcribed into cDNA by using a QuantiTect® reverse transcription kit (Qiagen, Germany). The cDNA was quantified by Picodrop (Pico 200, Cambridge, UK) and quantitative real-time PCR was performed using Power Sybr Green Master Mix (Applied Biosystem, Warrington, UK) in Step One Plus real-time detection system (Applied Biosystem, USA). The house keeping gene *rpsl* of *P. aeruginosa* was used as an internal standard [26]. ACT-2 positive isolate showing resistance to broad spectrum cephalosporins and also devoid of other β-lactamases was selected for this study. A pair of primers used for amplification of ACT-2 includes: forward primer 5'-CGGTAAAGCCGATGTTGCG-3' and reverse primer 5'-GAGCAATGGCATCGCCACCGA-3'. PCR reactions were performed in triplicates for the isolate. The reaction was run under the following conditions: 95 $^0$C for 2 min, 32 cycles of 95 $^0$C for 20 sec, 51 $^0$C for 40 sec, 72 $^0$C for 1 min. The relative quantification of bla$_{ACT-2}$ at different antibiotics pressure was determined by the ΔΔCt method.

**Statistical analysis**

Statistically, the distinctions in the expression of ACT-2 mRNA expression when induced with a different β-lactam antibiotic at different concentration compared with ACT-2 expression from uninduced strain were resolved with the assistance of one-way ANOVA and Tukey-Kramer (Tukey's W) multiple comparison test using SPSS version 17.0. The difference was

considered statistically significant at both 5% and 1% level when p<0.05.

DNA fingerprinting by repetitive extragenic palindromic (REP) sequences PCR:

Typing of all *bla*ACT-2 producing isolates was done by REP PCR as described previously [27].

## RESULTS

Ninety-seven (55.11%) out of 176 non-repetitive clinical isolates of *P. aeruginosa* was confirmed for the production of AmpC β-lactamase by M3DET. PCR screening for β-lactamase genes and sequence analysis confirm the presence of ACT-2 allele among 12 strains of *P. aeruginosa* which were obtained from different clinical specimens (Table 1). These ACT-2 harboring isolates possessed 5 different ESBL genes that were encountered among 7 isolates; CTX-M (n=2), SHV (n=3), TEM (n=2), VEB (n=2) and OXA-10 (n=1). These ESBL determinants were detected in different combination, these include SHV-CTX-M (n=2), one each of TEM-CTX-M-OXA-10, TEM-CTX-M. None of the carbapenemase genes were detected among ACT-2 harboring isolates as shown in Table 2 although, the presence of other plasmid mediated AmpC genes CIT (n=2) and DHA (n=3) were detected in 5 isolates. Out of 12 ACT-2 harboring isolates, the inducible phenotype was observed among 9 isolates and fingerprinting by REP PCR revealed that the isolates were not clonally related. Antimicrobial susceptibility testing revealed that these *bla*ACT-2 harboring isolates showed high resistance to expanded-spectrum cephalosporins and even to carbapenem group of drugs tested (Table 2). It also shows moderate to high resistance against amikacin, gentamycin, co-trimoxazole, ciprofloxacin. A high MIC was noticed against all

tested cephalosporins: cefotaxime (128-≥512 mg/L), ceftriaxone (128-≥512 mg/L), ceftazidime (128-≥512 mg/L), monobactam: aztreonam ((128-≥512 mg/L) and including carbapenem group of drugs: imipenem (<2-64 mg/L), meropenem (<2-64 mg/L), ertapenem (<2-64 mg/L). The details of MICs for strain harboring *bla*ACT-2 are illustrated in (Table 2).

A transformation experiment could establish that ACT-2 was encoded in plasmid and transformants harboring ACT-2 determinant could successfully conjugate into recipient *Escherichia coli* strain B which was confirmed by PCR analysis. On performing Incompatibility typing it was established that most of the strains with ACT-2 were associated with L/M, FIA, FIB Inc group (Table 2). To demonstrate whether the ACT-2 expression is induced in the presence of different β-lactam at a different concentration, wild type *P. aeruginosa* strain BM-501 (Table 2) was selected and the fold increase in mRNA production was measured using primer extension analysis. Analysis of gene expression is complicated when several mechanisms affecting the same class of antibiotics are at work. Therefore, the strain harboring only ACT-2 allele was selected for expression study. It was observed that the relative increase in mRNA level of ACT-2 gene varies when induced with different cephalosporins. Although a significant increase in ACT-2 expression 483 fold was achieved when the strain was induced by ceftriaxone at 4μg/ml followed by ceftazidime at 8 μg/ml (31 fold) and cefotaxime 4 μg/ml (8 fold) when compared with uninduced strain. Statistically, the differences in the expression of ACT-2 were found to be highly significant (p-value <0.05; Table 3).

Table 1. Clinical history of ACT-2 harboring strains of *P. aeruginosa*.

| Serial no | Sample ID | Patient's sex and age | Wards | Type of clinical specimen |
|---|---|---|---|---|
| 1 | BM19 | F/45 | Orthopaedic | Pus |
| 2 | BM27 | M/10 | Opthalmology | Conjunctival scrapping |
| 3 | BM74 | M/8 | Surgery | pus |
| 4 | BM219 | F/45 | Obstetrics & Gynaecology | pus |
| 5 | BM240 | M/2.5 | Surgery | pus |
| 6 | BM322 | F/10 | Surgery | Urine |
| 7 | BM339 | F/20 | Obstetrics & Gynaecology | Pus |
| 8 | BM457 | F/25 | Obstetrics & Gynaecology | Pus |
| 9 | BM501 | M/21 | Surgery | Pus |
| 10 | BM517 | F/29 | Surgery | Pus |
| 11 | BM539 | F/19 | Surgery | Urine |
| 12 | BM544 | F/19 | Surgery | Pus |

Table 2. Molecular details and resistance profile of ACT-2 allele-positive Pseudomonas aeruginosa isolates.

| Serial no. | Other β-lactamase genes detected | Location (Inc type) | Inducible phenotype detected | Resistance profile | MIC range of AmpC positive isolates against different beta-lactam drugs (µg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CTX | CRO | CAZ | FEP | ATM | IMP | MEM | ERT |
| 1 | DHA | - | Inducible | AMK, GEN, SXT | >512 | >512 | 128 | 64 | 256 | <2 | <2 | <2 |
| 2 | SHV | K | - | CIP, AMK, GEN, SXT | 512 | 512 | 128 | 32 | 256 | 8 | 4 | 8 |
| 3 | SHV | FIB | Inducible | CIP, AMK, GEN, SXT | 128 | 128 | 256 | 64 | 512 | <2 | <2 | <2 |
| 4 | CIT | | Inducible | CIP, AMK, GEN, SXT | 128 | 512 | 128 | 32 | 128 | 32 | | |
| 5 | DHA, SHV, CTX-M | FIA, FIB | Inducible | CIP, AMK, GEN, SXT | 256 | 512 | 256 | 16 | 256 | 16 | 8 | 16 |
| 6 | TEM, CTX-M, OXA-10, | L/M, K | Inducible | CIP, GEN, SXT | >512 | >512 | 512 | 64 | >512 | 32 | 64 | 32 |
| 7 | CIT, SHV, CTX-M | - | - | CIP, AMK, GEN, SXT | 512 | 256 | 512 | 32 | 256 | 64 | 64 | 64 |
| 8 | CTX-M, TEM | FIA, L/M | Inducible | CIP, AMK, GEN, SXT | 256 | 256 | 512 | 128 | 256 | 64 | 64 | 32 |
| 9 | - | - | Inducible | CIP, AMK, GEN, SXT | 128 | 128 | 256 | 16 | 256 | <2 | 8 | <2 |
| 10 | DHA, VEB | - | - | CIP, AMK, SXT | 512 | 512 | 512 | 32 | 512 | 8 | 16 | 16 |
| 11 | - | - | Inducible | CIP, AMK, GEN, SXT | >512 | 512 | 256 | 32 | 512 | 32 | 64 | 64 |
| 12 | VEB | - | Inducible | CIP, AMK, GEN, SXT | 512 | 256 | 256 | 16 | 128 | 16 | 4 | 8 |

Table 3. Statistical analysis of changes in ACT-2 mRNA expression in response to different β-lactam antibiotic stress at different concentration using one way ANOVA.

| Serial no. | Concentration of antibiotics | Value (Mean ± SEM) | | | |
|---|---|---|---|---|---|
| | | β-lactam antibiotics | | | |
| | | cefotaxime | ceftriaxone | ceftazidime | cefoxitin |
| 1 | NA | 1.00 ± 0 | 1.00 ± 0 | 1.00 ± 0 | 1.00 ± 0 |
| 2 | 2 µg/ml | 0.798 ± 1.37 | 10.326 ± 14.13 | 0.026 ± 0.01 | 1.548 ± 0.59 |
| 3 | 4 µg/ml | 7.558 ± 3.38 | 483.112 ± 12.05 | 0.011 ± 0.01 | 0.161 ± 0.06 |
| 4 | 8 µg/ml | 0.176 ± 0.28 | 186.186 ± 12.38 | 31.439 ± 9.70 | 0.653 ± 1.04 |
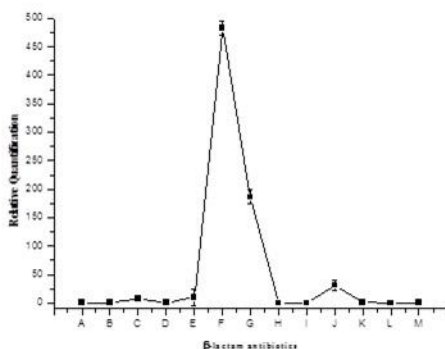


A=uninduced control, B=cefotaxime (2 µg/ml), C=cefotaxime (4 µg/ml), D=cefotaxime (8 µg/ml), E=ceftriaxone (2 µg/ml), F=ceftriaxone (4 µg/ml), G=ceftriaxone (8 µg/ml), H=ceftazidime (2 µg/ml), I=ceftazidime (4 µg/ml), J=ceftazidime (8 µg/ml), K=cefoxitin (2 µg/ml), L= cefoxitin (4 µg/ml), M= cefoxitin (8 µg/ml)

Figure 1. Transcriptional analysis of ACT-2 under different antibiotic exposure. Total bacterial RNA was isolated from mid-log-phase cultures of *Pseudomonas aeruginosa*. The error bars represent the standard deviations of the means of triplicate samples.

## DISCUSSION

Since the 1980s, only a few plasmids mediated AmpC β-lactamases have been reported in a strain of *P. aeruginosa* worldwide. The most widespread among them are those derived from chromosomal cephalosporinases of *Citrobacter freundii* [28,29]. Although documentation of EBC family (ACT/MIR) that was believed to be originated from AmpC genes of *Enterobacter spp.* in this strain is very limited and early detection is crucial for the proper implementation of antibiotic therapy. The first ACT-type ampC β-lactamase (ACT-1) was described in a strain of *Klebsiella pneumoniae* lacking a major outer membrane protein and confers resistance to carbapenem [9]. Its derivate ACT-2 was recently reported in a strain of *Enterobacter asburiae* (AM076977) and E.coli [13] from Italy and Morocco since then there was no report throughout the world. Though this β-lactamase was not well characterized its impact on antimicrobial therapy may be highly significant. The present study demonstrates the carriage of an ACT-2 allele in 12 strains of *P. aeruginosa*. To our knowledge, this is the first detection of ACT-2 in south central Asia and third report worldwide and strains harboring this β-lactamase were highly resistant to broad spectrum cephalosporin. This similar resistant pattern was observed in a strain harboring ACT-1 β-lactamase [9]. It is not unusual to find a single isolate that expresses multiple β-lactamase enzymes, thus present study also investigates the coexistence of different β-lactamase enzymes in clinical isolates of *P. aeruginosa*. These isolates co-harbor ESBL genes which include CTX-M, SHV, TEM, VEB and OXA-10 along with other plasmid encodes AmpC β-lactamases (CIT and DHA) which is commonly reported among enterobacterial isolates [30] but not in *P. aeruginosa* and its multiple detections of β-lactamase in the present study are alarming. The present study demonstrates that the ACT-2 allele was encoded on a plasmid and could conjugatively transfer ACT-2 allele, as ACT alleles were known to be located on both chromosome and plasmid [9)]. Typing by PCR reveal that ACT-2 was associated with Inc FIA, FIB, L/M, K although the earlier study report that the EBC allele (ACT-1) was carried in Inc L/M [31]. Higher expression of β-lactamases by pathogens during treatment with β-lactams is of particular concern, especially for pathogens with a high minimum inhibitory concentration (MIC) leading to treatment failure [32-35]. This overexpression of β-lactamase may be due to inappropriate antimicrobial dosing or the types of antibiotics the pathogen is getting exposed [36, 37]. Present study observed an increase in expression of ACT-2 in response to different cephalosporin's stress. However, the significant increase in expression of ACT-2 was observed when the strain was induced with ceftriaxone at 4 µg/ml followed by ceftazidime at 8 µg/ml. This overexpression of ACT allele, when induced with β-lactam antibiotics was supported well by an earlier study, though they do not establish the concentration of antibiotics at which higher expression occurred [12].

### Conclusion

This study highlights the prevalence of ACT-2 plasmid mediated AmpC β-lactamase along with other β-lactamase genes in clinical isolates of *P. aeruginosa* representing a serious therapeutic challenge. Therefore, early detection of these resistance mechanisms will improve surveillance studies, infection control, and available therapeutic options. Overexpression of ACT-2 in response to different cephalosporin stress requires re-evaluation of the treatment option with special consideration on appropriate dose available for the patients infected with this pathogen.

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