Occurrence of $\text{bla}_{\text{DHA-1}}$ mediated cephalosporin resistance in *Escherichia coli* and their transcriptional response against cephalosporin stress: a report from India

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Abstract

Background: Treatment alternatives for DHA-1 harboring strains are challenging as it confers resistance to broad spectrum cephalosporins and may further limit treatment option when expressed at higher levels. Therefore, this study was designed to know the prevalence of DHA genes and analyse the transcription level of DHA-1 against different β-lactam stress.

Methods: Screening of AmpC β-lactamase phenotypically by modified three dimensional extract method followed by Antimicrobial Susceptibility and MIC determination. Genotyping screening of β-lactamase genes was performed by PCR assay followed by their sequencing. The $\text{bla}_{\text{DHA-1}}$ transcriptional response was evaluated under different cephalosporin stress by RT PCR. Transferability of $\text{bla}_{\text{DHA}}$ gene was performed by transformation and conjugation and plasmid incompatibility typing, DNA fingerprinting by enterobacterial repetitive intergenic consensus sequences PCR.

Results: 16 DHA-1 genes were screened positive from 176 *Escherichia coli* isolates and primer extension analysis showed a significant increase in DHA-1 mRNA transcription in response to cefotaxime at 8 µg/ml (6.99 × 10² fold), ceftriaxone at 2 µg/ml (2.63 × 10³ fold), ceftazidime at 8 µg/ml (7.06 × 10³ fold) and cefoxitin at 4 µg/ml (3.60 × 10⁴ fold) when compared with untreated strain. These transcription data were found significant when analyzed statistically using one way ANOVA. Four different ESBL genes were detected in 10 isolates which include CTX-M (n = 6), SHV (n = 4), TEM (n = 3) and OXA-10 (n = 1), whereas, carbapenemase gene (NDM) was detected only in one isolate. Other plasmid mediated AmpC β-lactamases CIT (n = 9), EBC (n = 2) were detected in nine isolates. All DHA-1 genes detected were encoded in plasmid and incompatibility typing from the transformants indicated that the plasmid encoding $\text{bla}_{\text{DHA-1}}$ was carried mostly by the FIA and L/M Inc group.

Conclusion: This study demonstrates the prevalence of DHA-1 gene in this region and highlights high transcription of DHA-1 when induced with different β-lactam antibiotics. Therefore, cephalosporin treatment must be restricted for the patients infected with pathogen expressing this resistance determinant.

Background

*Escherichia coli* (E. coli) possess a chromosomal cephalosporinase gene, which is regulated by a weak promoter and a transcriptional attenuator. The gene confers resistance only to narrow-spectrum cephalosporins [1, 2]. However, spontaneous mutations in the promoter, as well as transcriptional attenuator region of the AmpC gene may induce constitutive overproduction of the cephalosporinase resulting in resistance to penicillins and broad-spectrum cephalosporins (e.g. cefotaxime, ceftazidime, ceftriaxone, aztreonam etc.) [3, 4]. Besides hyper-production of the chromosomally encoded enzyme, the presence of one or more plasmid-mediated AmpC β-lactamases along with other intrinsic mechanisms in *E. coli* leads to resistance
against multiple antimicrobial agents, compromising the efficacy of treatment [5–7]. Six families of plasmid-encoded AmpC β-lactamases were described based on their sequence similarities as CIT, FOX, MOX, DHA, EBC, and ACC [8]. The most commonly recognized plasmid-mediated AmpC among the strains of E. coli includes the CMY-2 type which belongs to the CIT family [9, 10].

DHA-1, another plasmid-mediated AmpC β-lactamase, belonging to DHA family was found increasingly among Enterobacteriaceae in many parts of the world and was a growing concern in the medical world as it leads to treatment failure [7]. It was first characterized in a Salmonella enteritidis which has the ability to hydrolyze penicillins, cephamycin, including broad spectrum cephalosporin leaving physicians with limited antibiotic choices. It was also the first plasmid-encoded β-lactamase found to be inducible and can be expressed in high levels [11, 12]. So far a total of 24 gene types of DHA family have been reported (http://www.ncbi.nlm.nih.gov/projects/pathogens/submit_beta_lactamase). The regulation of this β-lactamase expression is closely linked to cell wall recycling and involves at least three genes: ampR (codes for a transcriptional regulator of the LysR family), ampG (codes for a transmembrane permease) and ampD (codes for a cytosolic N-acetyl-α-hydroxymuramyl-l-alanine amidase) [13].

Though it was well established that β-lactam antibiotics are potent inducers of class C in most of the members of the family Enterobacteriaceae [7], there is no relevant information on the level of AmpC expression taking place when the strains with incomplete regulatory elements were under antibiotic stress. Therefore, this study was undertaken to investigate the transcriptional response of DHA-1 under various cephalosporin’s stresses.

Methods
Bacterial strains
A total of 176 consecutive, non-duplicate Escherichia coli isolates were collected from different clinical specimens (mostly from urine followed by pus) obtained from different Wards/OPD of Silchar Medical College and Hospital, India from October 2012 to March 2013. The isolates were identified by cultural characteristics, biochemical reactions and further confirmed by 16S rDNA sequencing using primers, a forward primer 5′-AGAGTTTGATCMTGCGCTCAG-3′ and a reverse primer 5′-TACGGYTACCTTGTTACGACTT-3′.

Screening of AmpC β-lactamase by cefoxitin disc test and modified three dimensional extract method
Preliminary screening of AmpC β-lactamase was carried out on Mueller–Hinton Agar plates containing cefoxitin (30 μg) (Hi Media, Mumbai). Isolates with inhibition zones of less than 18 mm, were considered as screen positives [14]. The suspected AmpC β-lactamase producers were further confirmed by modified three dimensional extract test (M3DET) [15]. Escherichia coli ATCC 25922 and Enterobacter cloacae P99 were used as negative and positive control respectively.

Antimicrobial susceptibility and minimum inhibitory concentrations (MIC’s) determination
Antimicrobial susceptibility was determined by Kirby Bauer disc diffusion method on Mueller–Hinton Agar plates. Following antibiotics were used: amikacin (30 μg), gentamicin (10 μg), ciprofloxacin (30 μg), trimethoprim/sulphamethoxazole (1.25/23.75 μg), tigecycline (15 μg) (Hi Media, Mumbai). MIC’s of various antibiotics were also determined on Mueller–Hinton Agar plates by agar dilution method according to CLSI and EUCAST guidelines [16, 17]. Following antibiotics were used: cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, ertapenem and aztreonam (Hi-Media, Mumbai, India).

Detection of DHA gene by polymerase chain reaction
Polymerase chain reaction (PCR) was performed targeting all the DHA genes by using a pair of primers as listed in Table 1. Isolates positive for DHA genes were further investigated for the presence of other AmpC gene families, namely: CIT, ACC, FOX and EBC [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 s, 51 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 7 min.

PCR products were purified by QIAquick Gel Extraction Kit (QIAGEN, Germany) and sequenced. Sequence results were analysed using a BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Molecular characterization of ESBL and carbapenemase genes by multiplex PCR
For amplification and characterization of ESBL genes, a set of five primers were used, namely: TEM, CTX-M, SHV, OXA-2, and PER [19]. Reactions were run under the following conditions: initial denaturation at 94 °C for 5 min, 33 cycles of 94 °C for 35 s., 51 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min.

For amplification and characterization of carbapenemase genes, a set of seven primers were used, namely: KPC, IMI, NMC, SME, VIM, IMP and NDM (Table 1). Reactions were run as described previously.

Transcriptional expression analysis of blaDHA-1 by quantitative realtime PCR
Expression of the blaDHA-1 gene was studied in response to cefoxitin, cefotaxime, ceftriaxone and ceftazidime
stress at different concentrations (2, 4, 8 µg/ml) and was determined by inoculating the organisms harboring \( \text{bla}_{\text{DHA}} \) in Luria–Bertani broth (Hi-media, Mumbai, India). Isolate without any antibiotic pressure was used as a control. A total RNA was isolated using Qia-gen RNase Mini Kit (Qiagen, Germany), immediately reverse transcribed into cDNA by using 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 \( rpsel \) of \( \text{E. coli} \) was used as an internal standard [25]. DHA-1 positive isolates showing resistance to broad spectrum cephalosporins and also devoid of other β-lactamases was selected for this study. The primer used for amplification of DHA-1 is listed in Table 1. PCR reactions were performed in triplicates for the isolate. The reaction was run under the following conditions: 95 °C for 2 min, 32 cycles of 95 °C for 20 s, 48 °C for 40 s and 72 °C for 1 min. The relative expression of \( \text{bla}_{\text{DHA}} \) at a different antibiotics pressure was determined by the ΔΔCt method. Relative quantification was compared with strain grown for 16 h without any antibiotic pressure.

### Statistical analysis

The changes in DHA-1 mRNA expression in response to different β-lactam antibiotic stresses at different concentration were analyzed using one-way ANOVA followed by Tukey–Kramer (Tukey’s W) multiple comparison test using SPSS version 17.0. Differences were considered statistically significant at both 5 and 1% level when \( p < 0.05 \).

Data are presented as mean fold change + standard error of the mean.

### Plasmid preparation

The bacterial isolates were 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 \( \text{bla}_{\text{DHA}} \) gene by transformation and conjugation

The transformation experiments were carried out by heat shock method [26] using \( \text{E. coli} \) DH5α as the recipient. Transformants were selected on cefotaxime (0.5 µg/ml) containing LB Agar plates.

Conjugation experiments were carried out between clinical isolates as donors and a streptomycin resistant \( \text{E. coli} \) strain B (Genei, Bangalore) as the recipient. An overnight culture of the bacteria was diluted in Luria–Bertani broth (Hi-Media, Mumbai, India) and was grown at 37 °C till the O.D. of the recipient and donor culture reached 0.8–0.9 at A600. Donor and recipient cells were mixed at 1:5 donor-to-recipient ratios and transconjugants were selected L.B Agar plates supplemented with cefotaxime (0.5 µg/ml) and streptomycin (600 µg/ml).

### Plasmid incompatibility typing

For detection of incompatibility group type of plasmid carrying \( \text{bla}_{\text{DHA}} \) PCR based replicon typing was carried out, targeting 18 different replicon types, to perform five multiplex and three simplex PCRs to amplify the FIA,
FIB, FIC, HI1, HI2, I1-lg, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons [27].

**DNA fingerprinting by enterobacterial repetitive intergenic consensus sequences PCR**

Typing of all *bla*<sub>DHA-1</sub> producing *E. coli* isolates was done by enterobacterial repetitive intergenic consensus (ERIC) PCR as described previously [28]. Isolates were put into cluster based on banding pattern and dendogram was prepared by NTSYS software.

**Results**

During the study period, a total of 176 *E. coli* isolates were obtained from different clinical samples. Among these, 110 (62.5%) were resistant to cefoxitin and 63 (35.8%) isolates were found to show AmpC activity by M3DET. By performing PCR, 16 isolates were detected for DHA genes and showed a sequence identical to that of DHA-1 (Table 1). These isolates harboring DHA-1 gene were selected for further study. Among DHA-1 positive isolates four different ESBL genes were detected in 10 isolates which include CTX-M (n = 6), SHV (n = 4), TEM (n = 3) and OXA-10 (n = 1). Carbenapenemase gene (NDM-1) was detected only in one isolate. Other plasmid mediated AmpC β-lactamase CIT (n = 9), EBC (n = 2) were detected in nine isolates that carried either CTX-M (n = 3),SHV (n = 1), TEM (n = 1), NDM (n = 1) alone or CTX-M plus SHV (n = 2), CTX-M plus TEM (n = 1) and OXA-10 plus SHV (n = 1) (Table 2). These isolates harboring AmpC β-lactamase were mostly obtained from Surgery and medicine ward. To demonstrate whether DHA-1 expression would take place in the presence of different cephalosporins at a different concentration, an *E.coli* strain BM-567 (Table 2) harboring only DHA-1 β-lactamase and showing resistance to broad spectrum cephalosporins was selected. The fold increase in mRNA production was measured using primer extension analysis. It was observed that there was a significant increase in the expression of DHA-1 gene in response to cefotaxime, ceftriaxone, ceftazidime but not as high as those for cefoxitin when compared with the basal level without antibiotic pressure (Fig. 1). Though increased in transcription was observed in response to these β-lactam antibiotics, high transcript level were achieved when induced by cefotaxime at 8 µg/ml (6.99 × 10² fold), ceftriaxone at 2 µg/ml (2.63 × 10³ fold), ceftazidime at 8 µg/ml (7.06 × 10³ fold) and cefoxitin at 4 µg/ml (3.60 × 10⁴ fold) (Fig. 1). The ANOVA and Tukey–Kramer (Tukey’s W) multiple comparison test for checking the differences in the expression of DHA-1 was found to be significant (p value is less than 0.05; Table 3).

Typing by ERIC-PCR confirmed 16 different haplotypes (Fig. 2) indicating the diversity of the isolates. The susceptibility pattern of these *bla*<sub>DHA-1</sub> harboring isolates showed resistance towards β-lactam including broad spectrum cephalosporin but most of them were susceptible against a carbapenem group of drugs. They also show susceptibility to tigecycline and moderate to high resistance against amikacin, gentamycin, co-trimoxazole, ciprofloxacin. The MICs of selected β-lactam antibiotics for all the parental strains harboring DHA-1 were found to be above breakpoint level (Table 2). The transformation experiment could establish that DHA-1 was encoded in plasmid however, conjugation experiment revealed that only 4 isolates could conjugatively transfer DHA-1 gene in *E. coli* strain B which was confirmed by PCR analysis. On performing incompatibility typing it was established that most of the transformants with DHA-1 were associated with K, FIA, L/M, FIB, HI1, B/O & 11 Inc group (Table 2).

**Discussion**

The first plasmid mediated AmpC β-lactamase, to be reported was CMY-1, in 1989 [29]. Since then, several plasmid-encoded AmpC β-lactamases (ACC, FOX, MOX, CMY, ACT, etc.) have been reported in several genera of bacteria, including *Salmonella* spp., *Pseudomonas* spp., *Proteus mirabilis* and *Klebsiella pneumoniae* [7]. Among them plasmid encoded DHA-1; a clinically important AmpC β-lactamase was the first β-lactamase found to be inducible and can be expressed at higher levels in strains having AmpR regulatory gene [11, 30]. This plasmid mediated β-lactamase is now being increasingly detected in a strain of *E. coli* worldwide [31–33] and early detection of this β-lactamase (DHA-1) is mandatory for better antibiotic therapy and also to prevent further spread. The present study reports the prevalence of DHA-1 (9%) among *E. coli* strains in this region which is quite high compared to other studies [30, 32, 33] and typing of these DHA-1 harboring isolates by ERIC PCR revealed diverse haplotypes, indicating the spread of the DHA-1 gene through horizontal transfer. Based on the present susceptibility data (Table 2) and previous studies [11, 12], carbapenem and other non-β-lactam antibiotics such as tigecycline could be better drugs of choice for the treatment of infections caused by *E. coli* producing DHA-1.

From the earlier study, it appears that *E. coli* lack one of the regulatory component (AmpR gene), which leads to the lower level, non-inducible expression of AmpC [34]. However, inducible cephalosporinase (*bla*<sub>CMY-13</sub>) found associated with an AmpR gene was detected recently in a strain of *E. coli* [35]. Several broad spectrum cephalosporins were believed to increase the expression of AmpC β-lactamase [36], although the concentration which leads to increase in the expression of AmpC β-lactamase was not established.
Table 2: Clinical history, their molecular details and resistance profile of DHA-1 gene-positive *E. coli* isolates

| Sl. No. | Sample ID | Age (years) | Sex | Ward/clinics | Type of clinical specimen | ESBL genes detected | Carbapenemase genes detected | Other plasmid AmpC genes | Resistance profile | MIC of β-lactam (mg/l) |
|---------|------------|-------------|-----|--------------|--------------------------|---------------------|---------------------------|------------------------|------------------|------------------------|
|         |            |             |     |              |                          |                     |                           |                        |                  |                        |
| 1       | BM12       | 35          | Male| Surgery      | Pus                      | –                   | –                         | K                      | AMK, GEN, SXT    | >512 >512 256 >512 64 16 8 8 |
| 2       | BM26       | 107         | Female| Pediatrics | Urine                    | TEM                 | –                         | FIA                    | CIP, AMK, GEN, SXT | 64 128 64 8 64 <2 <2 <2 |
| 3       | BM59       | 55          | Female| Medicine    | Urine                    | –                   | –                         | FIA                    | CIP, AMK, GEN, SXT | 128 64 64 16 128 <2 <2 <2 |
| 4       | BM63       | 60          | Female| Surgery     | Pus                      | CTX-M               | –                         | CIT                    | CIP, AMK, GEN, SXT | 128 128 256 16 64 <2 <2 <2 |
| 5       | BM130      | 27          | Female| Surgery     | Pus                      | TEM                 | –                         | CIT, EBC               | CIP, AMK, GEN, SXT | >512 512 >512 64 256 16 4 4 |
| 6       | BM138      | 45          | Male| Surgery     | Pus                      | CTX-M               | –                         | –                      | CIP, GEN, SXT    | 64 128 128 32 128 <2 <2 <2 |
| 7       | BM197      | 30          | Female| Surgery     | Pus                      | –                   | –                         | CIT                    | L/M              | 512 >512 256 64 512 2 <2 <2 |
| 8       | BM230      | 43          | Female| Surgery     | Pus                      | CTX-M, SHV          | –                         | CIT                    | L/M              | 64 128 256 32 256 16 8 16 |
| 9       | BM252      | 7           | Female| Pediatrics | Urine                    | SHV                 | –                         | CIT, EBC               | F1B, FIA         | >512 >512 >512 128 256 8 <2 <2 |
| 10      | BM355      | 10          | Male | Medicine    | Urine                    | –                   | NDM                       | CIT                    | FIA              | >512 >512 >512 8 256 <2 <2 <2 |
| 11      | BM409      | 61          | Male | Medicine    | Urine                    | CTX-M               | –                         | CIT                    | K                | 64 64 32 16 128 <2 <2 <2 |
| 12      | BM441      | 40          | Male | Medicine    | Stool                    | CTX-M, SHV          | –                         | FIA                    | CIP, AMK, GEN, SXT | 32 64 128 32 256 <2 <2 <2 |
| 13      | BM508      | 48          | Male | Surgery     | Pus                      | –                   | –                         | CIT                    | I                | 32 32 32 16 256 <2 <2 <2 |
| 14      | BM520      | 55          | Female| Surgery     | Pus                      | OXA-10, SHV         | –                         | –                      | CIP, GEN, SXT    | 16 128 128 32 32 <2 <2 <2 |
| 15      | BM567      | 30          | Male | Medicine    | Urine                    | –                   | –                         | –                      | CIP, AMK, GEN, SXT | 128 256 256 32 256 <2 <2 <2 |
| 16      | BM576      | 40          | Female| Medicine    | Urine                    | CTX-M, TEM          | –                         | CIT                    | K, B/O           | >512 >512 >512 128 >512 16 16 32 |

AMK: amikacin; GEN: gentamicin; CIP: ciprofloxacin; SXT: cotrimoxazole; CTX: cefotaxime; CAZ: ceftazidime; CRO: ceftriaxone; FEP: cefepime; ATM: aztreonam; IMP: imipenem; MEM: meropenem; ETP: ertapenem

* Extended spectrum β-lactamase
This study demonstrates higher transcription of DHA-1 when induced with different cephalosporins. These differences in the relative amounts of RNA transcription of DHA-1 gene, when induced with different cephalosporins at a concentration below MIC level suggest that the transcription varies depending on the level of antibiotics stress. Higher AmpC production was supported by another finding, where \( \text{bla} \text{MIR-1} \), a plasmid-encoded AmpC gene exhibited a 95-fold increase in expression relative to WT \( \text{AmpC} \) [37]. Concentration dependent expression of AmpC cephalosporinase was also observed in a strain of \( \text{Pseudomonas aeruginosa} \), when the strain was induced with cefoxitin or clavulanic acid at 8, 16 and 50 \( \mu g/ml \) [38]. So far, the factors behind the quantitative differences of AmpC expression in \( \text{E.coli} \) strain when exposed to different \( \beta \)-lactam concentration is unknown. A transformation experiment could establish that all the DHA-1 gene were encoded in a plasmid which is in agreement with the previous study [12, 30–33] and Incompatibility typing from the transfectants indicated that the plasmid encoding \( \text{bla}_{DHA-1} \) was carried mostly by FIA and L/M Inc group as found in another study [39]. Although detection of other Inc group, namely HI1, FIB, II, K in the present study was mostly associated with CMY-2 and ACC harboring strains [39]. Plasmids carrying genes for AmpC \( \beta \)-lactamases often carry ESBL genes such as CTX-M [40, 41] as found in the present study, where most of these DHA-1 harboring isolates co-harbour ESBL genes (Table 2). Co-existence of New Delhi metallo-\( \beta \)-lactamase (NDM) gene was also observed in one isolate as the high prevalence of the \( \text{E. coli} \) harboring a metallo-\( \beta \)-lactamase known as the NDM has been increasingly observed in the Indian subcontinent [42].

Fig. 1 Transcriptional analysis of DHA-1 expression by RT-PCR. Total bacterial RNA was isolated from mid-log phase cultures of \( \text{E. coli} \). The error bars represent the standard deviations of the means of triplicate samples.
Conclusion
Strains harboring plasmid mediated AmpC (DHA-1) genes are often resistant to multiple antimicrobial agents and the overexpression of this resistant determinant when induced with different cephalosporins stress will further limit treatment option. The present study demonstrates that higher expression of DHA-1 takes place when induced with specific concentrations of β-lactam antibiotics, although further research is required to understand the factors behind the upregulation of DHA-1 gene in the future. Therefore, revision in cephalosporin usage policy is required for effective treatment of patients infected with pathogen harboring this mechanism.

Authors’ contributions
BI performed the experimental work, data collection and analysis and prepared the manuscript. AB supervised the research work and participated in designing the study and drafting the manuscript. DP and APM participated in sample collection and part of experiments. DB participated in statistical analysis. DD and AC Participated in experiment designing and manuscript correction. All authors read and approved the final manuscript.

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Competing interests
The authors declared that they have no competing interests.

Availability of data and materials
All the relevant data and information are presented in the manuscript.

Consent for publication
All the authors read and approved the final version of the manuscript.

Ethical approval
The work was approved by Institutional Ethical committee of Assam University, Silchar vide Reference Number: IEC/AUS/C/2014-003. The authors confirm that participants provided their written informed consent to participate in this study.

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