Transcriptome analysis of beta-lactamase genes in diarrheagenic Escherichia coli

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Beta (3)-lactamases are the most important agents that confer drug resistance among gram-negative bacteria. Continuous mutations in β-lactamases make them remarkably diverse. We carried out the transcriptome analysis of 10 β-lactamase genes of Extended-Spectrum β-lactamases (ESBL), Metallo β-lactamases (MBL), and AmpC β-lactamases (ABL) in drug-resistant and sensitive diarrheagenic E. coli (DEC) isolates obtained from children up to 5 years of age. Out of the 10 β-lactamase genes, four belonged to ESBL (TEM, SHV, CTX, and OXA); three to MBL (NDM-1, IMP, and VIM); and three to ABL (ACT, DHA and CMY) class of genes. The different categories of DEC were estimated for β-lactamases production using a set of conventional phenotypic tests, followed by detection of their messenger RNA (mRNA) expression. The study revealed a direct correlation between mRNA expression of these genes and the presence of antibiotic resistance; also corroborated by mutation analysis of the AmpC promoter region. All the 10 β-lactamase genes showed a significant increase in their expression levels in resistant isolates, compared to those of the sensitive isolates, indicating their possible role in the disease pathogenesis. Increase in mRNA expression of β-lactamase genes, and thereby virulence, may be due to multifactorial parameters causing phenotypic as well as genotypic changes. Our study highlights the necessity of instantaneous detection of β-lactamase gene expression to curb the overwhelming threat posed by emergence of drug resistance amongst the commensal E. coli strains in children from developing countries for larger public health interest.

Widespread infections caused by antibiotic-resistant microbes are the biggest threats to public health in developing countries. The most widespread class of human antibacterial is the β-lactams. The development of resistance to β-lactam antibiotics in gram-negative pathogens, especially in Escherichia coli (E. coli), is a result of the production of β-lactamase enzymes, which endows the microbes with the ability to hydrolyze the β-lactam ring1. So, these β-lactamases are the real “Achilles heel” of antibiotic resistance in bacteria, killing thousands of people across the world every year2.

Extended-Spectrum β-lactamases (ESBLs) and plasmid-borne AmpC β-lactamases (ABLs) both can hydrolyze penicillin, cephalosporins, and β-lactams; whereas ABLs have a broader substrate profile, can degrade cephamycins3 and are resistant to β-lactamase inhibitors. Clavulanic acid (CA) inhibits ESBL but not ABLs4. The resulting β-lactam-resistant phenotype in E. coli is mainly a consequence of the acquisition of plasmid-mediated β-lactamases such as class A - ESBL (TEM, SHV, CTX-M and OXA), class C plasmid-mediated AmpC (ACT, CMY and DHA) or by the hyperproduction of the chromosomal AmpC enzyme, and class A, B or D carbapenemases5,6. Metallo β-lactamases (MBLs) - VIM, IMP, and NDM, reported from Enterobacteriaceae in the recent past, further limits the treatment options7.

In spite of the lower occurrence of the plasmid-mediated ABLs compared to ESBLs, they have been reported widely from different areas of the world. Carbapenemases are the β-lactamases that include MBLs and serine β-lactamases (KPC, OXA, GES, etc.). The MBLs require zinc ion for their action, and these are inhibited by metal chelators like EDTA and thiol-based compounds but not by sulbactam, tazobactam, and clavulanic acid. Chromosomal or plasmid-mediated genes are responsible for MBL production, and these genes can be transferred to other gram-negative bacteria through horizontal gene transfer8.

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The expression levels of β-lactamases in gram-negative bacteria are reported to be lower in comparison to gram-positive bacteria\(^5,6\). The presence of mutations in the promoter region is the most common mechanism responsible for hyperproduction of β-lactamases\(^7-14\). In clinical isolates, the overexpression of the chromosomal AmpC enzyme is primarily linked to AmpC promoter mutations\(^15,16\). In E. coli, the strength of the AmpC promoter generally defines the level of transcription of the AmpC gene\(^11,16\). The E. coli promoters harbor two hexamers (35 and 10 regions) of conserved sequences which play an essential role in gene transcription. The 35-consensus sequence is TTGACA, and the 10-consensus sequence is TATAAT; together they constitute the Pribnow box. The sequences that are closer to the consensus make stronger promoter. Overexpression of the AmpC gene can result in resistance to ampicillin, cefoxitin and expanded-spectrum cephalosporins. These mutations are not always redundant and sometimes can be misinterpreted or pass undetected.

The drug resistance genes have the potential to be used as important molecular markers for analyzing the prevalence and effect of the developed resistance. As reviewed elsewhere, the resistance mediated by β-lactamases is of particular concern because third-generation cephalosporins or higher generation antibiotics against β-lactams have long been used to treat E. coli infections successfully\(^17\). Further, there is a limited data available regarding the presence of mutations in the promoter region is the most common mechanism responsible for hyperproduction of β-lactamases in diarrheagenic E. coli (DEC) from children in developing countries, particularly India. All this demands effort to further understand the mechanisms involved in epidemiology of β-lactamase mediated resistance and specific resistant genotypes, both locally and globally. Due to limitations in phenotypic methods for perceiving antibiotic resistance, highly sensitive and efficient molecular methods are serving as the promising tools to detect bacterial mRNAs\(^9,10\). Gene expression in bacteria is complicated because of unstable nature and short half-lives of the bacterial mRNA as compared to eukaryotic mRNA\(^2\), limiting the detection rate, accuracy and approach of phenotypic detection to all species of gram-negative microorganisms\(^2,4\).

As data regarding expression of β-lactamase genes in E. coli isolates from children in India; we in this study intended to target 10 β-lactamase genes for expression analysis with an aim to predict a real-time scenario of the DEC response under antibiotic stress. We investigated the effect of antibiotic treatment on ESBL, MBL and ABL genes in resistant and sensitive isolates of DEC to analyze the differences in transcriptomes and also understand the survival of multidrug resistant bacteria. The findings will be useful in enabling clinicians to prepare specific strategies for surveillance and prevent development of drug resistance in children from developing countries, like India, for larger public health interest.

**Result**

We in our previous study found that the most frequent category of DEC detected in paediatric population suffering from diarrhea was Enteropathogenic E. coli (EPEC) followed by Enteraggregative E. coli (EAEC), Enterotoxigenic E. coli (ETEC) and Enterohemorrhagic E. coli (EHEC)\(^2\). Forty E. coli isolates each from children with diarrheal symptoms not receiving antibiotics (group 1); children receiving antibiotic therapy for 72 hours or more for reasons other than diarrhea (group 2); and healthy children (group 3) were studied for identification of the DEC isolates. The number of DEC isolates detected in each study group were 40, 39 and 27, respectively.

**Antibiotic susceptibility testing.** Antibiotic susceptibility testing was performed on 16 antibiotic agents (Supplementary File - Table S1). It was found that highest resistance rate was observed in cefotaxime (55.83%) followed by gentamicin (25.83%), ampicillin (25%), norfloxacin (21.66%), amikacin (19.1%), piperacillin/tazobactam (17.5%) and imipenem (15%). Cefazidime, ciprofloxacin, aztreonam and nalidixic acid showed similar antibiotic response (9% to 11.6%). In group 1 and 2, highest frequency of resistance was seen with cefotaxime in 67.5% and 82.5% isolates, respectively, while in group 3 it was highest with norfloxacin (25%).

**Quantitative reverse transcription Real-Time PCR (RT-qPCR) melting curve analysis.** Individual analysis of the genes showed symmetrical peaks with some exception where frequently asymmetric peaks were observed. Each target gene, from DEC isolates, after PCR amplification presented with different melting curves and distinct Tm values during melting analysis. The melting temperature (Tm) was as follows: TEM: 82.55 ± 0.50; SHV: 86.26 ± 0.65; CTX: 85.01 ± 0.25; OXA: 80.15 ± 0.62; IMP: 87.93 ± 0.37; NDM-1: 78.01 ± 0.56; VIM: 82.29 ± 0.67; ACT: 82.35 ± 0.58; CMY: 85.08 ± 0.32; and DHA: 78.10 ± 0.66 (Supplementary File - Figs S1–S3). The same Tm, for each gene, was detected with the positive control strains. Remarkably, little variation in Tm was observed among the different strains tested.

**Nucleotide accession numbers.** Nucleotide sequences were compared against GenBank database by using Basic Local Alignment Search Tool (BLAST). Sequence of β-lactamase genes and reference genes were submitted to NCBI, and accession number were obtained for TEM (KY941097, KY753820); CTX (KY753817, KY883447, KY883448, KY883449, KY883450); NDM-1 (KY753818); CMY (KY753819); glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-1 (KY775450); 16SrRNA (KY775448, KY775449, KY786039, KY786040, KY786041, KY786042, KY786043, KY786044, KY786045, KY786046, KY786047); ACT (KY883446); SHV (KY883445); and OXA (KY913604).

**Transcription analysis.** Fifteen DEC isolates from each group were studied for mRNA expression analysis. The relative expression of the related genes in 15 resistant isolates showed a high fold change (Fig. 1a–j). The level of expression in these isolates for TEM gene showed a range of 0.87–20.73; for SHV 1.43–18.03; for CTX-M 1.42–14.99; for OXA 1.41–14.99; for ACT 1.39–6.15; for CMY 0.44–13.56; for DHA 0.48–13.87; for NDM-1 0.92–14.00; for IMP 1.32–12.16; and for VIM 1.33–11.80-fold. The relative expression levels of these genes in sensitive isolates (Fig. 2a–j) showed a range of 0.43 to 1.48 for TEM gene; 0.34 to 1.49 for SHV; 0.56 to 1.49 for CTX-M; 0.42 to 1.65 for OXA; 0.11 to 1.59 for ACT; 0.29 to 1.75 for CMY; 0.33 to 1.57 for DHA; 0.23 to 1.28 for NDM-1; 0.29 to 1.50 for IMP; and 0.12 to 1.50 for VIM (Table 1). Average fold change in relative expression for each β-lactamase drug resistant and sensitive gene is shown in Supplementary File (Fig. S4). Both the internal control genes (16SrRNA...
and GAPDH] showed the stable level of expression at all the conditions. The mRNA transcripts for 16SrRNA and GAPDH genes gave similar signals for all isolates; however, different signals were obtained for different target genes. Overall, the mRNA expression of the target genes was found increased in the resistant isolates.

Data analysis. Significant differences were observed in the mean expression levels of the \( \beta \)-lactamases genes in resistant and sensitive DEC isolates by the \( 2^{-\Delta\Delta CT} \) method as shown in Fig. 3(a–j). The mean and standard
The error of the mean for all the genes were calculated. For TEM gene, resistant isolates presented a 6.89 ± 5.42 fold increase in the expression levels when compared to the sensitive group with 1.04 ± 0.36 fold expression (95% CI: 2.97 to 8.72, p < 0.001); similarly for SHV gene, a 6.31 ± 4.65 fold increase compared to 1.06 ± 0.387 (95% CI: 2.78 to 7.72, p < 0.001); for CTX-M gene, a 4.94 ± 4.08 fold increase compared to 1.09 ± 0.34 (95% CI: 1.68 to 6.01, p < 0.001); for OXA gene, a 4.47 ± 4.018 fold increase compared to 1.00 ± 0.38 (95% CI: 1.33 to 5.60, p < 0.001); for ACT gene, a 3.05 ± 1.65 fold increase compared to 0.95 ± 0.52 (95% CI: 1.18 to 3.01, p < 0.001); for

Figure 2. Relative expression of β-lactamase genes in drug-sensitive DEC isolates as determined by qPCR and analyzed by the 2^−ΔΔCt method. Box plots showing expression of (a) TEM gene, (b) SHV gene, (c) CTX-M gene, (d) OXA gene, (e) ACT gene, (f) CMY gene, (g) DHA gene, (h) NDM-1 gene, (i) IMP gene, (j) VIM gene for 15 sensitive DEC isolates.
Table 1. Details of the β-lactamase genes studied along with the number of resistant and sensitive isolates with N-fold <1 (decreased expression), N-fold ranging from 1 to 1.99 (comparable expression) and N-fold ≥2 (increased expression). The mean fold change in relative expression in both resistant and sensitive isolates is also shown. “Resistant; “Sensitive; “significant p-value; “only 6 resistant isolates were present.

| Beta-lactamase | Gene | Antibiotic Resistance | Mean fold increase in gene expression in beta-lactamase isolates |
|----------------|------|-----------------------|---------------------------------------------------------------|
|                |      |                       | OR (95% CI)                                                   |
|                |      | N (%)                 | N (%)             | N (%)          | N (%)          | p-value |
| ESBL           | TEM  | Cefotaxime            | 1 (3.33)          | 2 (6.66)       | 6 (20)         | 12 (40) | 0      | 6.89 ± 5.42 | 1.04 ± 0.36 | <0.001* | 5.85 (2.97–8.72) |
|                | SHV  | 0                     | 8 (26.6)          | 3 (10)         | 7 (23.3)       | 12 (40) | 0      | 6.31 ± 4.65 | 1.06 ± 0.39 | <0.001* | 5.25 (2.78–7.71) |
|                | CTX-M| 0                     | 8 (26.6)          | 4 (13.3)       | 7 (23.3)       | 11 (36.6) | 0      | 4.94 ± 4.08 | 1.09 ± 0.34 | <0.001* | 3.85 (1.68–6.01) |
|                | OXA  | 0                     | 10 (33.3)         | 4 (13.3)       | 5 (16.6)       | 11 (36.6) | 0      | 4.47 ± 4.02 | 1.00 ± 0.38 | <0.001* | 3.47 (1.33–5.60) |
| MBL            | NDM-1| Imipenem              | 1 (3.33)          | 10 (33.3)      | 3 (10)         | 5 (16.6) | 11 (36.6) | 0      | 5.09 ± 3.83 | 0.81 ± 0.39 | <0.001* | 2.95 (2.24–3.61) |
|                | IMP  | 0                     | 7 (23.3)          | 4 (13.3)       | 5 (16.6)       | 11 (36.6) | 0      | 2.96 ± 2.57 | 0.95 ± 0.40 | <0.001* | 2.81 (1.97–3.93) |
|                | VIM  | 0                     | 7 (23.3)          | 5 (16.6)       | 5 (16.6)       | 10 (33.3) | 0      | 3.68 ± 2.89 | 0.87 ± 0.43 | <0.001* | 3.20 (1.65–4.35) |
| ABL            | ACT  | Cefoxitin             | 0                 | 9 (30)         | 4 (13.3)       | 5 (16.6) | 11 (36.6) | 0      | 3.05 ± 1.65 | 0.95 ± 0.52 | <0.001* | 2.79 (1.91–3.70) |
|                | CMY  | 1 (3.33)              | 10 (33.3)         | 3 (10)         | 5 (16.6)       | 11 (36.6) | 0      | 3.66 ± 3.01 | 0.87 ± 0.53 | <0.001* | 2.79 (1.17–4.41) |
|                | DHA* | 1 (3.33)              | 19 (63.3)         | 2 (6.66)       | 5 (16.6)       | 3 (10)   | 0      | 6.14 ± 5.71 | 0.89 ± 0.41 | <0.001* | 5.25 (2.26–8.23) |

CMY gene, a 3.66 ± 0.01 fold increase compared to 0.87 ± 0.53 (95% CI: 1.17 to 4.41, p < 0.001); for DHA gene, a 6.14 ± 5.71 fold increase compared to 0.89 ± 0.41 (95% CI: 2.27 to 8.23, p < 0.001); for NDM-1 gene, a 5.09 ± 3.83 fold increase compared to 0.81 ± 0.39 (95% CI: 2.25 to 6.31, p < 0.001); for IMP gene, a 2.96 ± 2.57 fold increase compared to 0.95 ± 0.40 (95% CI: 0.63 to 3.39, p < 0.001); for VIM gene, a 3.68 ± 2.89 fold increase compared to 0.87 ± 0.43 (95% CI: 1.26 to 4.35, p < 0.001). Mean range fold change in β-lactamase gene expression in all the isolates is shown in Supplementary File (Fig. S5).

Analysis of promoter region. Sequence analysis of the AmpC promoter region obtained from the sequencing of PCR amplicons of fifteen resistant isolates showed base substitutions and base insertion (Fig. 4). A transversion of T → A at position −32 changed the wildtype −35 box (TTGTCGA) to the consensus −35 box sequences (TTGACA), causing overexpression of promoter (10 to 40-folds) when compared to ATCC 25922 (p < 0.05); and at position −11 C → T changed the wild-type −10 box from TACAT to TAAAT, causing 20-fold change in expression level (p < 0.05). Point mutations involving transversion of C → T at −42, −1 and +8 positions were also detected causing moderate level of β-lactamase production (1.5 to 8-fold). Apart from that, an insertion of T between −20 and −21 was also observed causing 4 to 6-fold change in gene expression level. Although insignificant, this insertion causes very low level of expression due to its presence in spacer region and it was rare. In sensitive samples, only one out of fifteen showed transversion at −42 position, which is a weak promoter. All these mutations in the AmpC coding region are the possible cause of overexpression of mRNA of these target genes in resistant isolates. The sequenced promoter region gene was compared in the GenBank database by BLAST and submitted to the NCBI database (Accession no. MK139014).

Discussion

Enterobacteriaceae have accumulated an extensive array of β-lactam genes over a period of several years encoding the ESBL, ABL, and more recently carbapenemases. We attempted to analyze, comprehensively, the changing paradigm of antibiotic resistance in infections caused by E. coli. Many genes are speculated to be involved in the complicated antibiotic resistance, but the data available is limited. We focused on this aspect to gain an understanding of the genes involved in antibiotic resistance.

The mRNA is synthesized only by viable cells and, therefore, it can be used as a marker of cell viability in bacteria. The mRNA is a highly unstable, fragile entity with a very short half-life and needs a harsh working environment and operating conditions. Hence, it is crucial to use the precise amount of mRNA with careful calculations to overcome its loss/compensation to obtain high and consistent yield with high purity. Extreme precautions are indispensable for sample preparation to maintain consistent levels of mRNA throughout the process and reduce variability in the results owing to the loss or degradation of the mRNA during preparation.

The mRNAs quantification by RT-qPCR is useful in relative comparison of resistance gene expressions. Internal controls serve to eliminate and normalize inter-sample variation during the isolation and reverse transcription steps involving mRNA. Furthermore, it helps to reduce variations in total transcriptional activity between cells.

The expression pattern of β-lactam genes for resistant and non-resistant isolates was analyzed in 15 samples each. The crucial step in the RT-qPCR assay is the variance in the percentage of transcribed mRNA into cDNA, as the efficiency of extracted mRNA can vary for the same targets. In between the resistant isolates, we noticed a difference in the levels of expression of all β-lactam genes. The mRNA expression of 10 β-lactam genes of ESBL, MBL, and ABL classes were found to be high in resistant isolates, except for few isolates where mRNA expression was either negligible (down-regulated) or comparable to the control strains. In the case of drug-sensitive isolates, the mRNA expression level was mostly found down-regulated, except for few isolates where it was comparable to...
the control strains, suggesting their non-pathogenic nature. However, down-regulation in sensitive strains could be a result of process of genetic gain or development.

Resistance to β-lactams due to the expression of various genes among DEC isolates has become a widespread phenomenon, and their expansion among other members of Enterobacteriaceae is increasing. Consequently, this

**Figure 3.** Box plots for β-lactamase (ESBL, MBL and ABL) genes for drug-resistant (R) and sensitive (S) DEC isolates analyzed by the $2^{-\Delta\Delta Ct}$ method. The relative expression levels of (a) TEM, (b) SHV, (c) CTX-M, (d) OXA, (e) ACT, (f) CMY, (g) DHA, (h) NDM-1, (i) IMP, (j) VIM genes for 15 DEC (each of resistant and sensitive) isolates is shown. The significance levels were derived by Student’s unpaired t-test. Error bars indicate standard deviation. ** Indicates $p$-value < 0.001 for all the genes.
from diarrhea or any other diseases were also enrolled. Each group consisted of 40 children.

Financial support

This work was supported by the Department of Science and Technology, Government of India.

Conflicts of interest

The authors declare no competing interests.

Ethical considerations.

The study was conducted on children up to five years of age with symptoms of diarrhea and not receiving antibiotics, and admitted children receiving antibiotic therapy (oral or I/V) for 72 hours or more for reasons other than diarrhea, attending out-patient department of a tertiary care hospital (University College of Medical Sciences and Guru Teg Bahadur Hospital, University of Delhi). Healthy children who were not suffering from diarrhea or any other diseases were also enrolled. Each group consisted of 40 children.

Ethical considerations. All the experimental protocols were approved by the Institutional Ethics Committee – Human Research (IEC-HR) of the University College of Medical Sciences and Guru Teg Bahadur Hospital.
Hospital, New Delhi. The methods were carried out in accordance with the relevant guidelines. Written informed consent was obtained from the parents/local guardians of children before their enrollment in the study.

**Sample collection and processing.** Fresh stool samples were collected in clean, leak-proof, well-labeled, sterile and wide-mouthed plastic containers and were transported immediately to the laboratory for culture. Up to five dark pink colonies (lactose fermentation) with the typical appearance of *E. coli* on MacConkey agar were selected and subjected to conventional biochemical tests for identifying *E. coli* such as gram staining (gram-negative and rod-shaped bacterium), catalase test (+ve), oxidase test (−ve), glucose fermentation with production of gas, fermentation of other sugars (lactose, sucrose, maltose and mannitol), nitrate reduction (+ve; reduces nitrate into nitrite), urease (−ve), Methyl Red Voges Proskauer [MR (+ve) and VP (−ve)], OF glucose test (glucose Fermenter), decarboxylase test [lysine (+ve), arginine (−ve) and ornithine (+ve/−ve)], indole test (+ve), Simon’s citrate (−ve) and hydrogen sulfide (−ve)40. PCR for 16SrRNA gene was also performed as an internal quality control for *E. coli* 41.

**Detection of diarrheagenic *E. coli* (DEC).** All the *E. coli* isolates were checked for the presence of virulence genes of DEC by conventional multiplex PCR method using the following set of genes: *elt* and *est* for ETEC; *eagg* and *east* for EAEC; *eae* for atypical EPEC, and *eae* + *bfp* (eaf) for typical EPEC; *stx* and *hyla* for EHEC; *ipah* for EIEC; and *daaE* for DAEC. Primers used for amplifying the sequences were based on previously published literature25. The constitutively expressed and highly conserved GAPDH gene in bacteria was used as control in RT-qPCR experiments42–44.

**Antibiotic susceptibility testing.** Antimicrobial susceptibility testing was performed with 16 antimicrobial agents (HiMedia Laboratories, Mumbai, India) as per CLSI guidelines45. The *E. coli* (ATCC) strain 25922 was included as a quality control.

**Identification of β-lactamase producing *E. coli*.** The criteria for determination of β-lactamase producing *E. coli* were defined as: the presence of TEM (Temoneira), SHV (Sulfhydryl variable),CTX (Cefotaxime hydrolyzing capabilities), and OXA (Oxacillin hydrolyzing capabilities) for ESBL; NDM-1 (New Delhi Metallo β-lactamase), IMP (Imipenem), and VIM (Verona integron-encoded Metallo-β-lactamase) for MBL; and ACT (AmpC type), CMY (Cephamycins), and DHA (Dhahran Hospital) for ABL class of genes. Primers were selected from previously published literature as shown in Table 241,46–50.

**RNA isolation and cDNA synthesis.** Total RNA was isolated from 2 ml of fresh overnight Luria-Bertani (LB) broth culture/peptone water from the stool samples of resistant (resistant to three or more antibiotics), non-resistant and control strains by manual trizol method (Invitrogen, India) according to the manufacturer’s instructions.
instructions. The RNA was treated with DNase-I (Invitrogen, India) to minimize the risk of DNA contamination. The quantity of extracted RNA was determined by A260 measurements, where reading of 1.0 equals to approximately 40μg of single-stranded RNA/μl. The purity (A260/A280) of RNA was >1.8 when measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, India). First-strand cDNA was prepared from 1μg of total RNA for all the isolates using 1μl of oligo (dT) primers, 2.5μl of 10x cDNA synthesis buffer (Thermo Scientific, India) in a 25μl of total volume to obtain 40 ng equivalent RNA/μl. The PCR conditions of 25 °C for 10 mins, 50 °C for 50 mins and 85 °C for 5 mins were used. The cDNA was diluted in the ratio of 1:10 using DEPC treated water and stored at −20 °C. The cDNA from _E. coli_ isolates from healthy children was used as negative control and was processed along with other resistant and sensitive isolates. The 16SrRNA and GAPDH genes were used as internal controls51–53. The intensity was expressed as a value relative to that of the 16SrRNA54.

Quantitative Real time PCR (qPCR). Fifteen isolates each of the resistant and sensitive _E. coli_ were taken up for relative quantification of the β-lactamases genes along with 15 control strains. The qPCR was performed using an SYBR Green qPCR kit (Roche Diagnostics, USA) and a Light Cycler 480-II system (Roche Diagnostics, USA) was used to measure the relative transcript levels of the 10 β-lactamase genes in resistant and non-resistant isolates. Initially, primers were standardized with cDNA using conventional PCR.

A total of 20μl reaction volume was used including 3μl cDNA, 10μl of (2x) Syber green master mix, 1μl each of forward and reverse primers (10μM) and nuclease-free water to make up the volume. No-template control (NTC) was run with each reaction for each gene. The NTC were PCR mixtures containing water in place of template cDNA. The genes were amplified with an initial denaturation of 95 °C for 5 mins, followed by 40 cycles of 95 °C for 20 secs., 55 °C or 60 °C for 30 secs., and 72 °C for 20 secs. High number of PCR cycles were run to ensure the saturation of all the reactions and to obtain a sufficient amount of product. The acquisition temperature for fluorescence was 72 °C for all the genes. The RT-qPCR was performed in triplicates to minimize any errors caused by handling and average of all the three values was taken as final.

To validate amplification specificity further, a single cycle of the melting curve was performed with 95 °C for 10 secs. and 65 °C for 20 secs. with the continuous acquisition (from 65 °C gradually increasing by 0.1 °C/sec. to 95 °C, with fluorescence data acquisition every 1 sec.) and finally cooling at 40 °C. The instrument automatically calculates melting curves by converting them into melting peaks by plotting the negative derivative of fluorescence measured at 533 nm and generates melting peaks by plotting about temperature (2dF/dT). The melting curves were calculated for all the isolates using qPCR. Relative gene expression (fold change) was calculated using the formula 2−ΔΔCt. Constantly expressed genes were used as internal controls in relative quantification studies. Threshold cycle value (Ct value) is defined as the PCR cycle where the fluorescence signal increases above the background threshold28.

Transcriptional assays. The greater the quantity of target cDNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower Ct56. The Ct values were read and recorded for ESBL, MBL and ABL, and the endogenous genes in resistant, non-resistant and control strains. An average of Ct values for all 15 controls in the control strains was used for normalization. The difference between Ct of the drug-resistant or drug-sensitive genes and the internal control genes gave the ΔCt values. Subsequently, ΔΔCt for all the resistant and sensitive DEC isolates were calculated, which is the difference between the ΔCt of the resistant or sensitive isolates and the ΔCt of the control strains. Relative quantification or the fold change in expression of the β-lactamase genes was calculated as N-fold which is equal to 2−ΔΔCt for all isolates versus the control strains59.

Sequencing and data submission. Sequencing of the β-lactamase genes (amplified by the primers shown in Table 2) was performed commercially (Helix Biosciences, Bangalore, India). To increase the accuracy of the results, sequencing was performed with both forward and reverse primers and sequences were compared against GenBank database by using BLAST and submitted to the NCBI database.

DNA sequence analysis of promoter region. DNA was extracted from _E. coli_ colonies by using the commercial kit (Real Biotech Corporation, Taiwan) following the manufacturer’s instructions. For AmpC promoter mutation analysis, a 271-bp fragment was amplified using a forward primer (5′-GATGGTCTGCGGCTGTG-3′) and a reverse primer (5′GGGAGCAAATGTGGAGCAA-3′)52. PCR amplicons were sequenced commercially (Helix Biosciences, Bangalore, India). All isolates were characterized genetically by mutational analysis. The promoter sequences were compared to the wild-type sequence from _E. coli_ (ATCC 25922), used as a promoter control by multiple sequence alignment using the BioEdit online tool (version 7.0.5).

Statistical analysis. Statistical analysis was done using SigmaStat statistical software package (SPSS). Student’s unpaired ‘t’ test was performed to analyze the data derived by the 2−ΔΔCt method. p-value of less than 0.05 was considered as significant. The transcript levels of the target genes for both resistant and sensitive isolates were presented as mean ± SEM (standard error of the mean).

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Acknowledgements
This work was supported in part by the Council of Scientific and Industrial Research, Library Avenue, Pusa, New Delhi -110012, India [Project: 08/532 (0007)/2011-EMR-I]. Special thanks to all the participants of this research. All staff members of our department are acknowledged for their support.

Author Contributions
T.S. and S.D. conceived and designed the study, analyzed/interpreted results. T.S. collected the samples. T.S., P.K.S., S.W. and S.A.D. performed experiments, collected and analyzed the data. T.S., S.W. and A.J. carried out the literature search. T.S., P.K.S. and S.W. gave technical support, and S.A.D. gave conceptual advice. S.D., T.S. and S.A.D. wrote the manuscript. A.J., S.W. and P.K.S. performed the manuscript editing. S.A.D. participated in the design and supervision of the study, and along with A.J. revised the final version of the manuscript. S.D. supervised the study and also revised the final version of the manuscript. All the authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-40279-1.

Competing Interests: The authors declare no competing interests.

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