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# Prevalence of multidrug-resistant *Salmonella typhi* in typhoid patients and detection of *blaCTX-M2* and *blaCTX-M9* genes in cefetoxime-mediated extended spectrum β-lactamase-producing *Salmonella typhi* isolates.

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## Abstract

**Objectives: Prevalence of multidrug-resistant (MDR) *S. typhi* in typhoid-positive patients, phenotypic screening and molecular detection of *blaCTX-M2*, and *blaCTX-M9* ESBL coding genes.**
**Materials and methods: Sixty-six of the 650 clinical blood samples collected have turned out to be positive for *S. typhi*. Antimicrobial susceptibility and MIC was done as recommended by CLSI. Phenotypic screening of ESBL production and the PCR amplification ESBL coding genes *blaCTX-M2*, and *blaCTX-M9* was also achieved.**
**Results: Sixty-six out of 650 clinical blood samples have turned out to be positive for *S. typhi* with an incidence rate of 10.15%. Among 66 isolates (n=44) isolates were MDR. The significant increase in MIC was observed between 128-256 µg/ml to cefetoxime, among the isolates, 9 *S. typhi* isolates reported positive for cefetoxime-mediated ESBL production. The PCR amplification of ESBL coding genes *blaCTX-M2* and blaCTXM-9 yielded amplicon size of 884 and 692 bp, respectively. The ciprofloxacin resistant and cefetoxime sensitive standard MTCC *S. typhi* 734 and *S. typhi* BST 63 strain showed absence of both *blaCTX-M2* and *blaCTXM-9* genes**
**Discussion: The study shows an increase in the incidence rate of MDR *S. typhi* infection and ESBL production in *S. typhi* clinical isolates.**

**Keywords:** *Salmonella typhi*, Multidrug resistant (MDR), ESBL, *blaCTX-M2*, *blaCTX-M9.*

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## Introduction

Majority of *Salmonella* sp. causes various types of clinical syndromes in humans and have been broadly categorized into two groups. The first, enteric fever, is caused by *Typhoidal salmonella* (TS) like *S. typhi* (typhoid fever), *S. paratyphi* A, and *S. paratyphi* B (Paratyphoid fever) and are mostly transmitted through contaminated water and food. The second type of clinical syndromes like diarrhea and gastroenteritis are caused by non-*typhoidal Salmonella serovars* [1]. In India, enteric diseases are most prevalent in urban areas, with the incidence approaching one percent of the population annually in some endemic areas [2]. Earlier studies have shown that majority of enteric fevers are prevalent in most of the developing countries like India, Pakistan, Bangladesh, and several African nations. In India, some reports suggest the incidence of typhoid fever in urban slum areas was as high as 0.2% (2 typhoid positive patients per 1000 population) per year among children less than 5 years of age, and 0.51% (5.1 typhoid positive patients per 1000 population) per year among those under 10 years of age [3]. Incidence of typhoidal infection varies with age. Studies from Kolkata have shown that the incidence of typhoid fever is higher in persons with average age of 14.7 y [4]. In contrast, the incidence of paratyphoid fever is higher among persons mean age of 17.1 y [4]. A study conducted in north India shows that incidence ratio of typhoidal fever is higher in children between 5 and 12 y of age, and the ratio is far higher in children aged less than 5 y; 24.8% of cases per year were reported among the children aged less than 5 y [5].

Extended Spectrum Beta-Lactamases (ESBL) were originally recognized in the year 1983 [6]. They belong to group of amber-class A β-lactamase enzyme that potentially hydrolyze β-lactam antibiotics with an oximinino group such as third-generation cephalosporin's and aztreonem, and are completely inactivated by β-lactamase inhibitors. The screening of the positive ESBL production in *S. enterica serovars* was detected and reported from various countries like Italy, France, and Nepal [7]. The significant increase in the production of CTX-M-β-lactamases, a family of ESBLs, preferably hydrolyze cefetoxime (CTX) has been recognized and reported with increasing frequency [8]. CTX-M type of ESBLs or cefotaximases encoded by *blaCTX-M* genes located in a plasmid or on the chromosome belongs to type of class A β-lactamases [9]. This widespread resistance mechanism across the most part of the globe, from Europe, Africa, Asia, South America, and most recently from North America with several reports of clinical isolates producing these lactamases [8,10].

The CTX-M-type ESBLs causing serious concerns by displaying significantly increased level of resistance to both cefetoxime (CTX) and ceftriaxone (CTR) than that to ceftazidime (Caz). Approximately, 40 various types of CTX-M enzymes have been identified, and classified into five groups based on their amino-acid sequence homology, subsequently, been reported in different enterobacteria [11]. The first cefotaximase to be reported in *Salmonella* sp. was CTX-M-2, subsequently, over ten other CTX-M enzymes have been identified and reported in different *Salmonella serovars* with *S. enterica serovar typhimurium* being the most frequent, producing CTX-M-type enzymes such as CTX-M-5 and CTX-M-15 [6,12,13].

Presently, ciprofloxacin is preferred as a drug of choice for the treatment of typhoid infections. Its frequent administration has resulted in dramatic emergence of *S. typhi* with reduced susceptibilities to fluoroquinolones [2]. A study conducted in Kolkata has given a strong evidence of fluoroquinolone-resistant *S. typhi* with the Minimum Inhibitory Concentration (MIC) as high as 16 µg/ml to ciprofloxacin and norfloxacin [14]. Fluoroquinolone resistance in India is similar in pattern to other Asian countries [15].

The principal objective of the conducted study was to determine the incidence of multidrug resistance (MDR) and screening of extended spectrum β-lactamase (ESBL) in *S. typhi* strains isolated from typhoid patients from Kalaburagi region, Karnataka, India, and PCR amplification of plasmid-encoded *blaCTX-M*2 and *blaCTX-M*9 genes majorly responsible for cefetoxime-mediated resistance in *S. typhi* isolates.

## Materials and Methods

### *Isolation of S. typhi from clinical samples*

In the present study, 650 blood samples were collected from typhoid-suspected patients confirmed by Widal test from Government Hospital, Kalaburagi, India and Pooja Diagnostic Centre, Kalaburagi, India during July 2013-December 2013. The samples were enriched in tryptic soy broth with 100 units of streptokinase to dissolve clot. They were processed and tested biochemically according to standard guidelines for *S. typhi* isolation [16]. The isolated *S. typhi* strains were screened in relation to Standard MTCC *S. typhi* 734 and genotypically confirmed by 16S rDNA sequencing at Chromous biotech laboratory, Bangalore, India, with universal primers.

### *Antimicrobial susceptibility testing*

The *S. typhi* isolates and standard MTCC *S. typhi* 734 were tested for antibiotic susceptibility and resistance for various groups of antibiotics by Kirby-Bauer disk diffusion method as per the standard guidelines of Clinical Laboratory Standard Institute (CLSI) [17,18]. The 16 antibiotic disks (Himedia Pvt. Ltd., Mumbai, India) selected for this study are listed in Table 1. The zone of inhibition post incubation was measured using standard antibiogram scale.

### *Determination of MIC*

MIC was determined by agar dilution method and broth dilution method following CLSI standard guidelines 2012 [18] of five selected antibiotics: ciprofloxacin, nalidixic acid, chloramphenicol, cefetoxime, ampicillin (commercially available as ciprofloxacin hydrochloride monohydrate), nalidixic acid-free acid, chloramphenicol, cefetoxime sodium salt, ampicillin sodium salt (Himedia laboratories, Mumbai, India). Stock solutions of the required antibiotics were prepared as follows: ciprofloxacin (2 mg/ml), nalidixic acid (2 mg/ml), chloramphenicol (1 mg/ml), cefetoxime (1 mg/ml), ampicillin (1 mg/ml) [19]. Significant MIC breaking points to the respective antibiotics were interpreted.

### *Screening of ESBL production in S. typhi (Extended spectrum ß-lactamases)*

**Combination disc method:** ESBL production in *S. typhi* isolates was determined by combination disc method with reference to Drieux et al. [20] on MHA (Muller Hinton Agar) medium seeded with *S. typhi* test organism. A combination of cephalosporin's/clavulanic acid, i.e. cefetoxime (30 µg) and ceftazidime (30 µg) in combination with clavulanic acid (30 µg +10 µg), was placed 30 mm apart as suggested in standard CLSI guidelines, 2012 [18] and incubated at 37°C for 24 h. A greater by 3 mm increase in zone diameter of either respective clavulanic acid combination discs versus its zone diameter when tested alone confirmed as a significant ESBL production.

**Double disc synergetic test:** Double disc synergy test is the confirmatory test to combination disc method and was performed by standard protocol of Drieux et al. [20]. This test was performed on MHA seeded with *S. typhi* isolates and impregnated with amoxiclav or augmentin (combination of amoxicillin with 10 µg of clavulanic acid) at the center surrounded by cephalosporin antibiotic discs like cefetoxime (30 µg), ceftazidime (30 µg), cefexime (30 µg) set 30 mm from the center (Standard CLSI Guidelines, 2012) and incubated at 37°C for 24 h. A significant enhancement in the inhibition zone with either one of the test antibiotics to Augmentin disc is confirms positive for ESBL production.

### *PCR detection of ESBL-resistant blaCTX-M2 and blaCTX-M9 genes*

PCR analysis was carried out to detect plasmid-encoded *blaCTX-M*2 and *blaCTX-M*9 responsible for cefetoxime-mediated drug resistance in *S. typhi*. The primers, sourced from Chromous Biotech Pvt. Ltd, Bangalore, India, were for *blaCTX-M*2, 5'-CGGTGCTTAAACAGAGCGAG-3' (Forward), *blaCTX-M*2 (reverse) 5' CCATGAATAAGC AGCTGATTGCCC-3', *blaCTX-M*9 (forward) 5'-GATTGACCGTATTGGGAGTTT-3', *blaCTX-M*9 (reverse) 5'-CGGCTG GGTAAAATAGGTCA-3' [21]. For PCR amplification, about 100 ng of DNA was added to 50 µl mixture containing 10 mM of dNTPs, 0.4 mM of each forward and reverse primer and 3 U of Taq polymerase in 10X PCR buffer containing 1.5 mM $MgCl_2$. Amplification was

performed in a Corbett CGI-96 thermocycler with cycling parameters comprising the following steps, initial denaturation at 94°C for 5 min, sequentially followed by, denaturation 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min for 35 cycles [22]. Finally, after accomplishing amplification, DNA ladder of 100 bp and 500 bp (Bangalore Genei Pvt Limited, India) were used to determine the size of amplicons.

## Results

Of the total 650 samples, only 66 have shown culture positivity for *S. enterica Serovar typhi* with isolation rate of 10.15%. Results of 16S rDNA sequencing show 100% resemblance with *S. typhi* isolates in nucleotide BLAST analysis in comparison with existing databases. The sequences have been submitted to National Centre for Biotechnology Information through bankit and the accession number of three MDR isolates, namely, BST 51 (accession no. KR537431) BST 103 (accession no. KR537432) and BST 130 (accession no. KR537433) were obtained from the existing database through BLAST and phylogenetic tree constructed using bioinformatics tool (Figure 1).

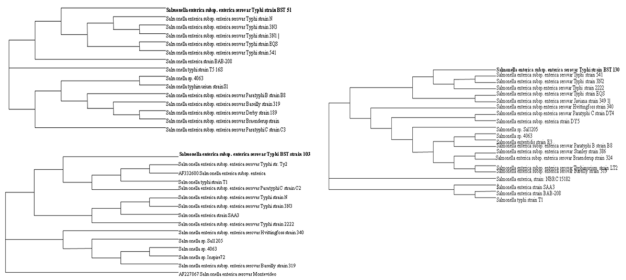

***Figure 1.*** *Phylogenetic tree constructed with significantly aligned 16S rDNA sequence of Salmonella typhi in BLAST search with query sequence of BST 51, BST 103 and BST 130.*

### *Antimicrobial susceptibility testing*

Sixty-six positive *S. typhi* isolates were chosen for this study. Among them, 06.06% (n=04) were ampicillin resistant but 100% (n=66) of isolates showed reduced susceptibility to aztreonem. In addition to that 24.25% (n=16) strains were resistant to cefexime, ceftazidime respectively, in contrast only 13.63% (n=09) to ceftriaxone. However, 46.97% (n=31) isolates were cefetoxime resistant, comparatively higher to other cephalosporin's. Subsequently, 42.42% (n=28), 46.97% (n=31) of isolates shown resistance to nalidixic acid, ciprofloxacin respectively in contrast 100% (n=66) isolates were showed reduced resistance against higher-generation quinolones such as levofloxacin and gatifloxacin. In addition to that, all n=66 isolates were entirely sensitive to cotrimoxizole and azithromycin. We observed decreased resistance in *S. typhi* isolates to aminoglycoside antibiotics; streptomycin and amikacin, i.e., 06.06% (n=04), 1.5% (n=01) respectively (Figure 2). Only 10.60% (n=7) isolates were resistant to tetracycline and 03.03% (n=2) to chloramphenicol. However, standard MTCC *S. typhi* 734 strain showed resistance to ciprofloxacin, in contrast it was susceptible to cefetoxime, cefexime and ceftriaxone belongs to group of cephalosporin's (Table 2).

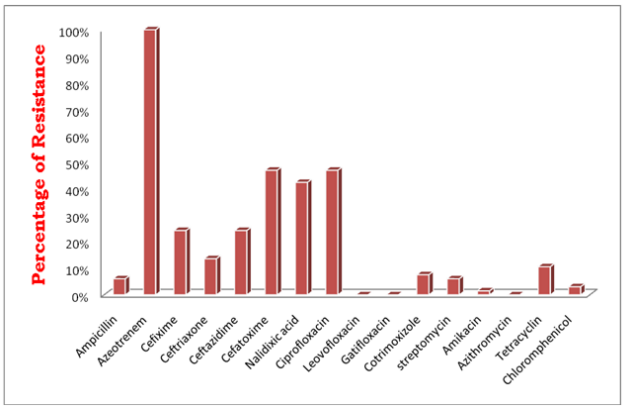


***Figure 2.*** *Graph showing the percentage of resistance against selected antibiotics by S. typhi isolates.*

### *MIC of S. typhi isolates*

Among n=31 ciprofloxacin-resistant strains; n=12 isolates showed a significantly increased MIC range of 128-256 µg/ml. Moreover, n=28 isolates have shown a remarkable increase in MIC range of 128-2048 µg/ml to nalidixic acid. However only one isolate *S. typhi* BST 174 has shown the maximum MIC of 512 µg/ml to chloramphenicol. Subsequently, strain *S. typhi* BST 43 has shown significantly increased MIC of 512 µg/ml to ampicillin and in contrast, *S. typhi* BST 45 and *S. typhi* BST 48 have shown MIC at 256 µg/ml. Consequently, n=4 strains, *S. typhi* BST 51, *S. typhi* BST 130, *S. typhi* BST 103, *S. typhi* BST 43, have shown a greater degree of MIC at 256 µg/ml to cefetoxime. In addition, the standard MTCC *S. typhi* 734 strain showed decreased MIC range to cefetoxime of 2 µg/ml and the high range of MIC of 128 µg/ml to ciprofloxacin (Table 3).

### *Phenotypic screening of ESBL production in S. typhi*

Of the 66 *S. typhi* isolates, 55% (n=36) showed resistance to third-generation cephalosporin's such as ceftazidime, ceftriaxone and cefetoxime. Among 36 cephalosporin's resistant *S. typhi* (CeRST) isolates, 25% (n=09) CeRST *S. typhi* isolates were positive ESBL producers. The ESBL production was evident in both combination disc method and double disc synergetic test. In addition to that entire n=9 *S. typhi* isolates were responsible for cefetoxime-mediated ESBL production (Figures 3a and 3b).

### *Detection of ESBL-resistant blaCTX-M2 and blaCTX-M9 genes*

All the n=9 cefetoxime-mediated ESBL-producing *S. typhi* strains have been selected for genotypic study, i.e., PCR amplification of cefotaximase genes, *blaCTX-M2* and *blaCTX-M9*. The samples showed amplification of 884 bp and 692 bp, respectively (Figures 4a and 4b), with primers specific to *blaCTX-M2* (accession no. KT277101) and *blaCTX-M9* (accession no. KT277102). The *blaCTX-M2* and *blaCTX-M9*

gene amplification was absent in ciprofloxacin resistant and cefetoxime sensitive standard MTCC *S. typhi* 734 and *S. typhi* BST 63.

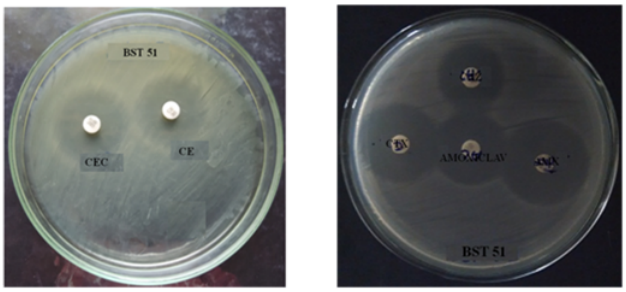


***Figure 3.*** *a. Increase in zone of inhibition with cefetoxime+clavulanic acid of ≥ 3 mm in comparison to cefetoxime alone. b. Enhancement in inhibition zone of cefetoxime, ceftazidime and cefexime towards β-lacatmase inhibitor clavulanic acid containing amoxiclav.*

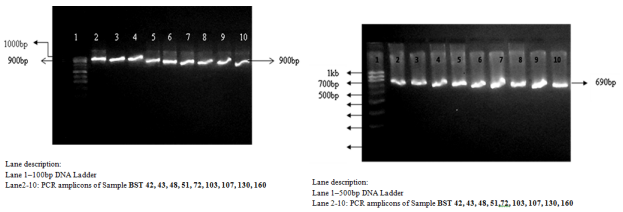


***Figure 4.*** *a. PCR amplification of CTX-M2 gene. b. PCR amplification of CTX-M9 gene.*

***Table 1.*** *Antibiotics selected for the study.*

| Sl. no | Antibiotic | Concentration (µg) |
|---|---|---|
| 1 | β-Lactams | |
| | Ampicillin | 10 |
| | Azeotrenem | 30 |
| 2 | Cephalosporins | |
| | Cefixime | 5 |
| | Ceftriaxone | 30 |
| | Ceftazidime | 30 |
| | Cefetoxime | 30 |
| 3 | Quinolones | |
| | Nalidixic acid | 30 |
| | Ciprofloxacin | 5 |
| | Levofloxacin | 5 |
| | Gatifloxacin | 5 |
| 4 | Cotrimoxizole | 25 |
| 5 | Aminoglycosides | |
| | Streptomycin | 10 |
| | Amikacin | 30 |
| 6 | Macroloides | |
| | Azithromycin | 15 |
| 7 | Tetracycline | 30 |
| 8 | Chloromphenicol | 30 |

***Table 2.*** *Antibiotic resistance of Salmonella typhi isolates.*

| Sl. no | Antibiotic | No. of resistant strains | Percentage of resistance (%) | Percentage of intermediates (%) | Percentage of susceptibility (%) |
|---|---|---|---|---|---|
| 1 | β-lactams | | | | |
| | Ampicillin | 4 | 6.06 | 4.54 | 89.39 |
| | Aztreonem | 66 | 100 | 0 | 0 |
| 2 | Cephalosporins | | | | |
| | Cefixime | 16 | 24.25 | 15.15 | 60.6 |
| | Ceftriaxone | 9 | 13.63 | 24.24 | 62.13 |
| | Ceftazidime | 16 | 24.25 | 28.78 | 46.97 |
| | Cefetoxime | 31 | 46.97 | 16.67 | 36.36 |
| 3 | Quinolones | | | | |
| | Nalidixic acid | 28 | 42.42 | 28.79 | 28.79 |
| | Ciprofloxacin | 31 | 46.97 | 13.64 | 39.39 |
| | Levofloxacin | 0 | 0 | 1.5 | 98.5 |
| | Gatifloxacin | 0 | 0 | 1.5 | 98.5 |
| 4 | Cotrimoxizole | 0 | 0 | 0 | 100 |

| | | | | | |
|---|---|---|---|---|---|
| 5 | Aminoglycosides | | | | |
| | Streptomycin | 4 | 6.06 | 21.2 | 72.73 |
| | Amikacin | 1 | 1.5 | 7.6 | 90.9 |
| 6 | Macroloides | | | | |
| | Azithromycin | 0 | 0 | 9.09 | 90.91 |
| 7 | Tetracycline | 7 | 10.6 | 7.6 | 81.8 |
| 8 | Chloromphenicol | 2 | 3.03 | 0 | 96.97 |

***Table 3.*** *Minimum inhibitory concentration (MIC) level of Salmonella typhi isolates.*

| S. no. | Antibiotics | MIC clinical breaking point (µg/ml) | MIC level (in µg/ml) | No. of resistant isolates |
|---|---|---|---|---|
| 1 | Ampicillin | 16-32 | 256-512 | 4 |
| 2 | Chloramphenicol | 8-32 | ≥ 512 | 2 |
| 3 | Cefotaxime | 8-32 | 16-256 | 31 |
| 4 | Nalidixic acid | 8-32 | 128-2048 | 28 |
| 5 | Ciprofloxacin | 1-4 | ≥ 128-256 | 31 |

## Discussion

Enteric fever has caused a major concern to developing countries like India, Pakistan, Nepal and African countries. The incidence of typhoid fever is higher in developing countries compared to developed ones. The prevalence of MDR *S. typhi* and ESBL-producing *S. typhi* has now become a challenging task to the field of medicine. Earlier, chloramphenicol was used extensively as a drug of choice to treat typhoid fever. Its extensive prescription triggered the emergence of chloramphenicol-resistant *S. typhi* in Aden, Chile, and Kuwait [23]. The first epidemic was caused by chloramphenicol-resistant *S. typhi* in the year 1972 in Mexico [23,24] and subsequent outbreaks were reported from Kerala, India [25], Vietnam, Indonesia and Korea [23,26]. In 1989, the first outbreak, caused by multidrug-resistant *S. typhi* (MDRST), which is resistant to chloramphenicol, ampicillin, trimethoprim, sulphonamides, and tetracycline, was reported from developing countries like India [26-29], Pakistan [30,31] and Bangladesh [32]. In 1991, ciprofloxacin has been recommended for the treatment of typhoid fever caused by chloramphenicol-resistant *S. typhi*. In 1992, chromosomal encoded resistance to ciprofloxacin with MIC range of 0.3 mg/L was reported [33].

The present study evaluates the incidence of MDR *S. typhi* and cefetoxime-mediated ESBL production in *S. typhi* strains isolated from Kalaburagi City, Karnataka, India. Of the total of 650 Widal-positive samples collected, n=66 have shown positivity for *S. typhi* at the incidence rate of 10.15%, which is significantly higher in comparison with earlier studies conducted in this area [34]. Sixty-seven percent (n=44) of isolates were MDR and have shown resistance to more than three antibiotics. Antibiotic susceptibility test has shown 46.97% (n=31) strains having reduced susceptibility to ciprofloxacin; in contrast, to all n=66 highly susceptible to third- and fourth-generation antibiotics like levofloxacin and gatifloxacin. Decreased resistance of 7.15% (n=02) was observed in ampicillin, chloramphenicol, streptomycin and 10.6% (n=07) in tetracycline, respectively. Altogether, 66 isolates have shown 100% susceptibility to both cotrimoxizole and azithromycin. Interestingly, 54.54% (n=36) have shown remarkably reduced susceptibility to third-generation cephalosporin's. The percentage of resistance is relatively higher in cefetoxime 86.11% (n=31), cefexime 44.44% (n=16), ceftriaxone 25% (n=9) and ceftazidime 44.44% (n=16) respectively.

Twelve out of 31 ciprofloxacin-resistant *S. typhi* isolates have shown an elevated level of MIC in the range of 128-256 µg/ml. There is a substantial increase in MIC level to cefetoxime with four (n=4) isolates in the range of 256 µg/ml; the result is relatively similar in comparison with the studies conducted in China by Yu et al. [35] on *S. typhimurium*. Four isolates have shown MIC to ampicillin in the range 256-512 µg/ml and also we found relatively higher range of MIC to chloramphenicol of 512 µg/ml.

Phenotypic detection of ESBL production was made against cefetoxime and ceftazidime by two standard phenotypic ESBL detection methods. Among n=36 CeRST isolates, 25% (n=9) strains have shown positivity for ESBL production; remarkably, all n=9 isolates were cefetoxime-mediated ESBL producers. Prevalence of ESBL production in India is far higher in comparison to the studies conducted in Nepal (0.5%) and Poland (0.3%) (36). ESBL production in *S. typhi* was genotypically confirmed by PCR amplification of *blaCTX-M*2 and *blaCTX-M*9 genes with amplicon size of 884 and 692 bp, respectively. The absence *blaCTX-M*2 and *blaCTX-M*9 amplification in cefetoxime sensitive standard MTCC *S. typhi*

733 and *S. typhi* BST 63 isolate indicates the significance of *blaCTX-M*2 and *blaCTX-M*9 genes in cefetoxime mediated ESBL production. The similar studies carried out by Xie et al. also showed the significance *blaCTX-M* genes in cefetoxime mediated drug resistance in *E. coli* isolates. Ryoo et al. [21] found nearly similar product length on *Klebsiella pneumonia*. Earlier studies already reported the presence of *blaCTX-M*2 gene in *S. typhimurium* and *S. oranienberg* [36-38].

## Conclusion

Our study shows that the significant increase in the level of drug resistance among *S. typhi* isolates to higher-generation antibiotics like cephalosporin's and higher-generation fluoroquinolones with higher level of MIC. Emergence of ESBL in *S. typhi* majorly in developing countries reflects poorly on sanitary conditions, inadequate medical attention, and contamination of water and food. Antibiotic susceptibility test and ESBL detection are recommended prior to treatment.

## Conflict of Interest

The authors declare of no conflict of interest in conducting this study.

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