# *Original Research Article*

**Molecular Detection of blaTEM, blaCTX-M and blaSHV Genes in Extended Spectrum β-Lactamase (ESBL) *Escherichia coli* from clinical samples**




## ABSTRACT

**Introduction:** Extended spectrum β-lactamases are the group of β-lactamase enzymes which confer resistance to the oxyimino-cephalosporins and monobactams.

**Study design:** Cross-sectional prospective study

**Place and Duration of Study:** This study was conducted over a period of 2 years (September 2018 to April 2020) at microbiology laboratory of Nepal Mediciti Hospital.

**Methodology:** Clinical samples were processed in microbiology laboratory and culture isolates were characterized by standard microbiological techniques following standard procedures**.** Antibiotic susceptibility testing was performed by modified Kirby-Bauer disc diffusion method as recommended by Clinical and Laboratory Standard Institute. Extended spectrum βlactamases were phenotypically confirmed by combined disc method. ESBL producing genes i.e. blaTEM,blaCTX-M and blaSHV were confirmed by PCR.

**Results**: Of the 1449 total *Escherichia coli* isolates, 323/1449(22.29%) isolates were multidrug resistance. Among total MDR *E.coli* isolates, 215/323(66.56%) isolates were ESBL producers. The maximum number of ESBL *E.coli* was isolated from urine 194(90.23%), followed by sputum 12(5.58%), swab 5 (2.32%), pus 2 (0.93%) and blood 2 (0.93%). Antibiotic susceptibility pattern of ESBL *E.coli* producers showed highest sensitivity towards tigecycline (100%) followed by polymyxin B, colistin and meropenem. Out of 215 phenotypically confirmed ESBL *E.coli*, only 186(86.51%) isolates were found to positive by PCR.The last 29(13.49%) were negative for any of the resistant genes. Among the ESBL genotypes, most common was blaTEM 118(63.4%) followed by blaCTX-M 68(36.6%).

**Conclusion**: The emergence of ESBL producing *E.coli* isolates with high antibiotic resistant rates to commonly used antibiotics and increased predominance of major gene types *bla*TEM is a serious concern to the clinicians as well as microbiologist. This study forwarded a real massage to all the clinicians for the emergence of XDR and PDR resistant bacteria and preservation of antibiotics for their proper use in near future, if past experience with MDR and ESBLs is any indicator.

**Aims:** This study was focused to find out the ESBL producing *Escherichia coli* and detection of TEM, SHV and CTXM genes by Polymerase Chain Reaction (PCR)

**Keywords**: *E.coli*, Extended spectrum β-lactamase, Multidrug resistant

## 1. INTRODUCTION

Extended spectrum β- lactamases (ESBLs) are plasmid mediated enzymes which belong to the group of β-lactamase enzymes, which are able to hydrolyze and develop resistance to 3rd generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefuroximeand cefepime) and monobactams (aztreonam), without affecting cephamycins (cefoxitin and cefotetan) or carbapenems (imipenem, meropenem, and ertapenem).[1],[2]

The first ESBL was detected from Germany in 1983 and in 1985 from France and at the end of 1980s,and the beginning of the 1990s from United States.[3] The emergence of New TEM and SHV enzymes are still existing in Europe, and found to have distinct epidemic clones, for example SHV-12 detected in *Escherichia coli* and *Klebsiella pneumoniae* isolates in Italy.[4] CTX-M-9 group are commonly found in isolates in Spain and CTX-M-3 enzymes have been identified the major strains in Eastern Europe, although variants of CTX-M group 1 (including the CTX-M-15 type) are the most widespread throughout Europe.[3],[4]

The rapid increase in extended spectrum β-lactamases with the existence of multidrug resistant organisms is a global problem. The prevalence of ESBL producing organisms is more than 20% in Asia and South Africa**.** The detection of major genes such as *bla*TEM,*bla*CTX-M and *bla*SHV in ESBL producing *E.coli* by molecular methods and their antibiotic resistance pattern can provide valuable information about their epidemiology and help in formulation of rational antimicrobial therapy.[5]

In Developing country like Nepal also due to the increasing incidence of ESBL producing *Escherichia coli*, the cost associated with the consequences also rises, so considers as an economic burden on the

patients both in community and in hospital. Therefore, this study was conducted with the objectives of studying the spectrum of MDR and ESBL *Escherichia coli* producing strains and detection of TEM,SHV and CTX-M genes by Polymerase Chain Reaction. Characterization of ESBL *Escherichia coli* at molecular level may be beneficial to analyze the root cause of ESBL pattern which may help to make a positive contribution to current understanding and knowledge of the situation caused by ESBL *Escherichia coli* producing strains and for the development of better treatment strategy and prevention of the disease.

## 2. MATERIALS AND METHODS

### 2.1 Sample processing and Identification of Organisms

A cross sectional prospective study was conducted in Microbiology Laboratory of Nepal Mediciti Hospital, Bhaisepati; Nepal from September 2018 to April 2020. A total of 16542 clinical samples sent to the microbiology laboratory were processed and cultured by standard microbiological techniques. The identification of bacterial isolates were carried out by cultural, morphological characters, Gram stain and appropriate biochemical tests (triple sugar iron, indole, citrate, urease and motility) following standard procedures**.**

### 2.2 Antibiotic Susceptibility Tests

Antibiotic susceptibility testing was performed by modified Kirby-Bauer disc diffusion method as recommended by Clinical and Laboratory Standard Institute.[6]The antibiotics used were amikacin (30µg), gentamycin (10µg), ciprofloxacin (30µg), ceftriaxone (30µg), cefotaxime (30µg), ceftazidime (30µg), nitrofurantoin (300µg), norfloxacin (10µg), nalidixicacid (30µg) ofloxacin (5µg), cotrimoxazole

(25µg),cefixime (5µg), cefepime (30µg), tigecycline (15µg), imipenem (10µg), meropenem (10µg), polymyxin B (300µg) and colistin (10µg). Plates were incubated aerobically at 37°C for 24 hours. Zone diameter in millimeters was measured and organisms were identified as sensitive, resistant and intermediate as per CLSI 2017 guidelines. *Escherichia coli* strain ATCC 25922 was used as control strain.

### 2.3 Screening of ESBL

The screening was done by disc diffusion technique using 3$^{rd}$ generation cephalosporins (ceftazidime, cefotaxime and ceftriaxone). Isolates resistant to more than one of these agents were identified as possible ESBL producers. [7]

### 2.4 Confirmation of ESBL

For confirmation, combined disc test was performed using Ceftazidime (30µg) alone and ceftazidime with clavulanic acid (30µg/10µg) and cefotaxime (30µg) and cefotaxime with clavulanic acid (30µg/10µg). A difference in zone of inhibition by ≥5mm of either of ceftazidime clavulanic acid with ceftazidime alone and cefotaxime clavulanic acid with cefotaxime alone was interpreted as confirmed ESBL. [7]

### 2.5 Gene Identification

From confirmed ESBL E.coli, plasmid DNA was extracted using alkaline hydrolysis method. These plasmid DNA served as a template for PCR amplification using blaTEM, blaCTX-M and blaSHV specific primers (Marcogen,Korea). For PCR amplification, 1.5µl plasmid DNA was added to 25 µl mixture containing 13 µl master mixture (Solis Biodyne,Estonia),10.5µl nuclease free water and 0.5µl each of reverse and forward primers**.** PCR was performed in 5 Prime/02 thermal cycler using optimized condition.

Bibby Scientific, U.K.using optimized condition. For *bla*TEM gene identification,initial denaturation at 94$^{o}$C for 5 minutes followed by 30 cycles of each of denaturation (95$^{o}$C for 45 seconds), annealing at (50$^{o}$C for 45 seconds), and extension at (72$^{o}$C for 30 seconds), and final extension at (72$^{o}$C for 10 minutes).For *bla*SHV and *bla*CTX-M genes,initial denaturation at 94$^{o}$C for 5 minutes followed by 30 cycles of each of denaturation (95$^{o}$C for 45 seconds), annealing at 56$^{o}$C for 45 seconds and 62$^{o}$C for 45 seconds respectively, and extension at (72$^{o}$C for 30 seconds), and final extension at (72$^{o}$C for 10 minutes).The amplified product was subjected to gel electrophoresis (2% gel stained with ethidium bromide) at 70v for 45 minutes.DNA ladder (100bp) was used to estimate the molecular weight of amplified products. After electrophoresis, gel doc system was used for photo documentation.

### 2.6 Control

For ESBL test, *Escherichai coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603) were taken as negative control and positive control respectively. Confirmed *Escherichia coli* strains harbouring blaTEM, *bla*SHV,*bla*CTX-M were taken as positive control and nuclease free water as negative control.

### 2.7 Statistical Analysis

Data were entered and percentage were calculated and analyzed by using Statistical Package for Social Science (SPSS) version 21.

## 3. RESULTS

1449 *E.coli* isolates were recovered from various clinical samples. The highest number of *E.coli* was isolated from urine followed by sputum, swab, pus, blood, fluid, foley's tip, vaginal swab, catheter tip,

BAL, biopsy, bile suction tube, CVP tip,ET tube. Of the 1449 total *E.coli* isolates, 323/1449(22.29%) isolates were multidrug resistance. Among total MDR *E.coli* isolates, 215/323(66.56%) isolates were ESBL producers. The maximum number of ESBL *Escherichia coli* was isolated from urine 194(90.23%), followed by sputum 12(5.58%), swab 5 (2.32%), pus 2 (0.93%) and blood 2 (0.93%) (Table 1).

Table 1. Distribution of ESBL *E.coli* from clinical samples

| Specimen | ESBL *E.coli* No (%) |
|---|---|
| Urine | 194(90.23%) |
| Sputum | 12(5.58%) |
| Swab | 5(2.32%) |
| Pus | 2(0.93%) |
| Blood | 2(0.93%) |
| Total | 215(100.0) |

Antibiotic susceptibility pattern of ESBL *E.coli* producers showed highest sensitivity towards tigecycline (100%) followed by polymyxin b, colistin and meropenem (Table 2).

Table 2: Antibiotic susceptibility pattern of ESBL *E.coli*

| Antibiotics | Antibiotic susceptibility rate No (%) ESBL *E.coli*(215) | |
|---|---|---|
| | Sensitive | Resistant |
| Amikacin(AK) | 197(91.6) | 18(8.4) |
| Gentamycin(G) | 180(83.7) | 35(16.3) |

| | | |
|---|---|---|
| Ciprofloxacin(CIP) | 125(58.2) | 90(41.8) |
| Ceftriaxone(CTR) | 0( 0.0) | 215(100) |
| Cefotaxime(CTX) | 1 | 214(97.3) |
| Ceftazidime(CAZ) | 0( 0.0) | 215(100) |
| Nitrofurantion(NIT)* | 182(93.8) | 12(6.2) |
| Norfloxacin(NX)* | 109(56.2) | 85(43.8) |
| Nalidixic acid(NA)* | 9(4.6) | 185(95.4) |
| Ofloxacin(OF)* | 91(46.9) | 103(53.1) |
| Tigecycline(TGC) | 215(100) | 0( 0.0) |
| Imipenem(IPM) | 148(68.8) | 67(31.2) |
| Meropenem(MRP) | 194(90.2) | 21(9.8) |
| Polymyxin B(PB) | 210 (97.67) | 5 (2.33) |
| Colistin(CL) | 211 (98.13) | 4 (1.86) |

*Used in urine culture

Two hundred fifteen ESBL *E.coli* isolates were confirmed by PCR using *bla*TEM, *bla*CTX-M and *bla*SHV specific primers. Out of 215 phenotypically confirmed ESBL *E.coli*,only 186(86.51%) isolates were found to positive by PCR (Table 3).

Table 3: Distribution of ESBL genotypes in *E.coli*

| ESBL genotypes | ESBL producing *E.coli* (n=186) No (%) |
|---|---|
| blaTEM | 118(63.4%) |
| blaCTX-M | 68(36.6%) |
| blaTEM + blaCTX-M | 39(20.96%) |
| blaSHV | 0(0) |

The last 29(13.49%) were negative for any of the resistant genes. Among the ESBL genotypes, most common was blaTEM 118(63.4%) followed by blaCTX-M 68(36.6%).The co-existence of blaTEM and blaCTX-M in ESBL producing *E.coli* was 39(20.96%).No ESBL *E.coli* isolates co-harbored blaSHV and blaTEM,blaCTX-M and blaSHV or all three genes at the same time.

## 4. DISCUSSION

Despite the discovery of antibiotics, emergence of MDR and ESBLs producing bacteria due to the extensive use of extended spectrum cephalosporins (ESCs) since early 1980's is a significant evolution in antimicrobial resistance. Several other factors including misuse of drugs, inappropriate antibiotic treatment, extensive use of antimicrobials also contributed to the emergence of drug resistant bacteria. The present study was conducted in the department of microbiology laboratory, Nepal Mediciti Hospital

during a period of September 2018 to April 2020 with the aim of understanding the antibiotic profile of MDR and ESBL producing *Escherichia coli.*

In this study, a significantly high number of *E.coli* isolates were recovered from urine (75.77 %). With respect to urinary tract infection, *E.coli* showed a significant resistance towards nalidixic acid, co-trimoxaloze, and 3rd generation cephalosporins. This co-relates with other study done in other parts of Nepal. [8], [9] However, 73% resistant to ceftriaxone were reported in *E.coli* isolates by Fanta et al., [10] The reason for third generation cephalosporins resistance may be due to the extensive use of drugs.[11]

In Urinary Tract Infection, highest sensitivity was found to nitrofurantoin (96.5%) followed by amikacin (80.7%) and gentamycin (73.9%). Various studies have been reported similar findings.[12], [13], [14] This may be due to the proper use of these drugs in UTIs cases since these are the first line drugs for UTIs.

The analysis of antibiotic susceptibility of *E.coli* isolated from sputum, blood, swab, pus demonstrated a significant degree of sensitivity towards tigecycline (100%) followed by colistin (98% to 100%), polymyxin B (97% to 100%), meropenem (91% to 96%) and imipenem (79% to 90%). Similar results were shown in other studies.[15], [16] It was found to be higher resistant pattern of cephalosporins (22% to 93%), fluoroquinolones (26% to 85%), aminoglycosides (8% to 59%) as compared to urine isolates. Several studies conducted in Nepal showed similar results.[13], [17] In contrast to this study, a slightly higher sensitivity was detected towards cephalosporins, fluoroquinolones, aminoglycosides by Bamford etal., [18] The increasing trends of drugs resistance towards internationally recommended first line drugs is a major concern worldwide [19] and are irrational used in public and private sectors.[20]

The prevalence of ESBL *E.coli* was (215/323) 66.56% which was alarming high. Several studies reported high prevalence i.e.40-70% of ESBL *E.coli* among MDR *E.coli.* [9], [14], [16], [21], [22] But the study conducted by Onyedibe, K et al.,[23] in 2018 observed only 18.6% ESBL prevalence in *E.coli* which is analogous to other study.[24] This is not similar with our study due to variation in geography, study design and selection of type of antimicrobial agents. The indiscriminate use of β lactam antibiotics leads to generation of selective pressures which have led to the selection of a variety of mutated forms of β lactamases. [25]

The analysis of antibiotic susceptibility profile of ESBL *E.coli* isolates documented higher sensitivity rates to tigecycline (100%), polymyxin B (100%), colistin (100%) followed by amikacin (91.6%), meropenem (90.2%) and imipenem (68.8%). Sensitivity to nitrofurantoin was 93.8% against ESBL producing *E.coli* isolates from urine. So, it is the drug of choice for treating infection caused by ESBL producing *E.coli.* The result of similar study conducted in India. [26], [27]

In this study, the overall prevalence of ESBL genes was 186 (86.51%).Which is similar to other findings reported by Dirar et al.,2020 [28] in Sudan**,** Ahmad et al.,2019 [29] in Iraq. PCR analysis revealed the presence of *bla*TEM, *bla*CTX-M and *bla*SHV genes in ESBL producing *E.coli* was 118 (63.4%), 68 (36.6%) and 0 (0) respectively**.**

In the present study, *bla*TEM was the most predominant genotype of ESBL among *E.coli* isolates. This study is well supported by Dirar et al., 2020 [28] in Sudan**,** Ahmad et al., 2019 [29] in Iraq, Noha et al., 2020 [30] in Upper Egypt, Majid et al., 2017 [31] in Iran, Pandit et al., 2020 [32] in Nepal, Michael et al., 2018 [33] in Iraq, Sahoo et al.,2019 [34] in India, Jena et al., 2017 [35] in India.

The presence of multiple genotypes in a single isolate might be the result of complex antibiotic resistance pattern.Our findings does not agree with the study of Kpoda et al., 2018 and Sadeghi et al., 2021 where they reported *bla*TEM and *bla* SHV type (24.6%) and (34.7%) respectively. [36], [37] Similarly, Lohani et al., 2019 reported CTX-M +TEM and CTX-M +SHV type (21.2%) and (19.2%) respectively.[38]

Regarding the *bla*SHVgene, no *bla*SHV type *E.coli* was detected in our study.This is similar to study done in Nigeria.[39] However, several findings in Nepal reported the prevalence of *bla*SHV gene.[38], [40]

## 5. CONCLUSION

In conclusion, the present study highlights the emergence of MDR and ESBL producing *E.coli* isolates with high antibiotic resistant rates to commonly used antibiotics and increased predominance of major gene types *bla*TEM is a serious concern to the clinicians as well as microbiologist. Since the spread of MDR and ESBL producing *E.coli* has been increasing rapidly worldwide including developing country like Nepal, treatment options for resistant bacteria have been increasingly sorted. In the present study, no resistance was documented to tigecycline, polymyxin B, and colistin suggesting the suitable drug of choice for treating ESBL producing *E.coli* causing life threatening infections. Therefore, molecular detection and identification of ESBL producing bacterial isolates should be essential at routine laboratory level.Of particular concern, our findings emphasizes the need for implementation of strict antibiotic policy,clinical care management and antibiotic stewardship program absolutely required in each and every health sectors to minimize the increasing trends of MDR and ESBL isolates by all concern authorities which will help in reduction of mortality of patients. This study forwards a real massage to all clinicians for the emergence of XDR and PDR resistant bacteria and preservation of antibiotics for their proper use in near future, if past experience with MDR and ESBLs is any indicator.

**Ethical Approval:**

The ethical approval was taken from the Ethical Review Board of Nepal Health Research Council (NHRC), Kathmandu, Nepal.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ESBL | Extended Spectrum β- Lactamase |
| MDR | Multi Drug Resistant |
| bla | β-lactamase coding gene |
| ATCC | American Type Culture Collection |
| CLSI | Clinical Laboratory Standard Institute |
| CTX-M | Cefotaximase,Munich |
| TEM | Temoniera gene |
| SHV | Sulfhydril variable |