Original Research Article

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

ABSTRACT

Aims: This study was focused to find out the antibiotic susceptibility pattern of ESBL *Escherichia coli* and ESBLs producing genes.

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 indetified and 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 beta-lactamases 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 *E.coli* isolates, 323/1449(22.29%) isolates were multi -drug resistance.Among total MDR Escherichia 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%).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.

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

1. INTRODUCTION

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

In 1983, the first ESBL was detected from Germany 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 identifiedthe 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 beta 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 considered as an economic burden on the patients both in community and in hospital set up. Therefore, this study was conducted with the objectives

of studying the spectrum of MDR and ESBL *Escherichia coli* producing strains and molecular characterization of these resistant genes. 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. The ethical approval was taken from the Ethical Review Board of Nepal Health Research Council (NHRC), Kathmandu, Nepal. 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 3rd 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 negativecontrol and positive control respectively.Confirmed *Escherichia coli* strains harbouring *bla*TEM, *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 calculation were analyzed by using Statistical Package for Social Science (SPSS) version 21.

3. RESULTS

1449 Escherichia coli isolates were recovered from various clinical samples.The highest number of Escherichia 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 multi -drug resistance.Among total MDR Escherichia 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).

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

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).

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 has 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,*Escherichia coli* showed a significant resistance towards nalidixic acid, co-trimoxaloze,and 3rd generation cephalosporins.

In Urinary Tract Infection,highest sensitivity was found to nitrofurantoin (96.5%) followed by amikacin (80.7%) and gentamycin (73.9%). 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%). 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. The prevalence of ESBL *E.coli* was (215/323) 66.56% which was alarming high.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* isolated form urine. So, it is the drug of choice for treating infection caused by ESBL producing *E.coli.* In this study, the overall prevalence of ESBL genes was 186 (86.51%). 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. The presence of multiple genotypes in a single isolate might be the result of complex antibiotic resistance pattern.Regarding the *bla*SHVgene, no *bla*SHV type *E.coli* was detected in our study.

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 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.

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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)

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)

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)

LIST OF ABBREVIATIONS

ESBL	Extended Spectrum Beta 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