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Molecular Detection of BlaTEM, BlaCTX-M and BlaSHV Genes in Extended Spectrum β-Lactamase (ESBL) *Escherichia coli* from Clinical Samples

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Abstract

Background: Extended spectrum β-lactamases are the group of beta-lactamase enzymes which confer resistance to the oxyimino-cephalosporins and monobactams. Emergence of ESBL producing genes which possess a serious threat for the treatment of infections both in community and hospitals since it is found to be increasing trends of multidrug resistance. This study was focused to find out the ESBLs producing genes.

Methods: This was a cross-sectional study conducted over a period of 2 years (September 2018 to April 2020) at microbiology laboratory of Nepal Mediciti Hospital. Clinical samples were processed in microbiology laboratory and culture isolates were identified 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 MDR and ESBL producing *E.coli* isolates with high antibiotic resistant rates to commonly used antibiotics and increased predominance of major gene type's *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

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

Introduction

Extended-spectrum beta-lactamases (ESBLs) are the group of beta-lactamase enzymes, which hydrolyze and cause resistance to the oxyimino-cephalosporins (cefotaxime,ceftazidime,ceftriaxone,cefuroxime and cefepime) and monobactams(aztreonam), but not the cephamycins (cefoxitin and cefotetan) or carbapenems (imipenem, meropenem, and ertapenem), produced by *Escherichea coli* and *Klebsiella pneumonia* [1].

Emergence of resistant bacteria worldwide as a threat to favorable outcomes of treatment of common infections in community and hospital settings. *E.coli* is one of the commonest pathogen to exhibit multidrug resistance. Important risk factors for infection with MDR and ESBL *Escherichia coli* are prolonged antibiotic exposure, overstay in hospital, increased use of third generation cephalosporins, severe illness, increased use of intravenous devices or catheters [2].

The first ESBL was identified from Germany in 1983, from France in 1985, from United States at end of 1980s, and the beginning of the 1990s [3]. New TEM and the SHV enzymes are still emerging in Europe, and distinct epidemic clones have been found, for example E. coli and K. pneumoniae isolates with SHV-12 in Italy [4]. Isolates with the CTX-M-9 group are common in Spain and strains with the CTX-M-3 enzymes have been described chiefly in Eastern Europe, although clones producing the CTX-M group 1 (including the CTX-M-15 type) are the most widespread throughout Europe [5-7].

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 [8].

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.

Materials and Methods

Sample processing and Identification of Organisms

A cross sectional 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 bacterialisolates were carried out by cultural, morphological characters, Gram stain and appropriate biochemical tests (triple sugar iron, indole, citrate, urease and motility) following standard procedures.

Antibiotic Susceptibility Tests

Antibiotic susceptibility testing was performed by modified Kirby-Bauer disc diffusion method as recommended by Clinical and Laboratory Standard Institute. 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), polymyxinb (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 2013 guidelines. *Escherichia coli* strain ATCC 25922 was used as control strain.

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 [9,10].

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 [11].

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°C for 5 minutes followed by 30 cycles of each of denaturation (95°C for 45 seconds), annealing at (50°C for 45 seconds), and extension at (72°C for 30 seconds), and final extension at (72°C for 10 minutes). For *bla*SHV and *bla*CTX-M genes, initial denaturation at 94°C for 5 minutes followed by 30 cycles of each of denaturation (95°C for 45 seconds), annealing at 56°C for 45 seconds and 62°C for 45 seconds respectively, and extension at (72°C for 30 seconds), and final extension at (72°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.

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 *bla*TEM, *bla*SHV, *bla*CTX-M were taken as positive control and nuclease free water as negative control.

Statistical Analysis

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

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).

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

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

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

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 (Tables 2 and 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

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)

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

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 2017 to April 2019 with the aim of understanding the antibiotic profile of MDR and ESBL producing *Escherichia coli.*

The present study documented that the highest number of *E.coli* isolates were recovered from urine (n=1098(75.77).With regard to urinary tract infection, *E.coli* showed great extent of resistance to nalidixic acid, co-trimoxazole and third generation cephalosporins. Similar pattern of resistant in urinary isolates of *E.coli* was shown in Nepal and India [12-14]. In contrast to our result,

Perez et.al reported *E.coli* isolates were 94% resistant to ceftriaxone [15]. This may be due to the irrational use of third generation cephalosporins [16]. However; a significant degree of susceptibility was found to nitrofurantoin (96.5%) followed by amikacin (80.7%) and gentamycin (73.9%). Similar findings have been reported in various studies [11-14,17,18]. This may be due to the rational use of these drugs in UTIs cases since it is reserved drug for UTIs.

In this study, analysis of antibiotic susceptibility of *E.coli* isolated from sputum, blood, swab, pus demonstrated a significant degree of susceptibility towards tigecycline (100%) followed by colistin (98% to 100%), polymyxinb (97% to 100%), meropenem (91% to 96%) and imipenem (79% to 90%).Similar results were shown in other studies[14,19] 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 [12,19,20]. In contrast to our study, Bamford et al. noted higher susceptibility pattern towards cephalosporins, fluoroquinolones, aminoglycosides [21]. The increased level of drug resistance is a major concern worldwide since these are the first line drugs recommended internationally [22,23]. and are irrational used in public and private sectors [24,25].

The present study noted (323/1449)22.29% MDR *E.coli* isolates that were suspected of being ESBL producers were confirmed by combined disc method. 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* [11,19,26-29]. Kashyap et.al reported 37% ESBL *E.coli* [30]. But the study conducted by Anil chander et.al in 2013 observed only 13.51% ESBL prevalence in *E.coli* which is analogous result to other study [31,32]. This is not similar with our study due to the variation in geography, study design and selection of type of antimicrobial agents. The indiscriminate use of beta-lactam antibiotics leads to the generation of selective pressures which have led to the selection of a variety of mutated forms of beta lactamases [33]. Antibiotic profile of ESBL producing *E.coli* were found to be higher sensitivity towardstigecycline(100%), polymyxin B (100%), colistin(100%) followed by amikacin(91.6%), meropenem(90.2%)and imipenem(68.8%). Susceptibility 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.* The result of similar study conducted in Nepal and India [8,14,34]. High resistant rates were observed to cephalosporins, nalidixic acid followed by fluoroquinolones. Similar findings were reported by Al-Zarouni et. Al [35].

In the present study, out of 323 MDR *E.coli* isolates, ESBL *E.coli* phenotypes were found to be positive in 215(66.56%) isolates. Similar findings were reported by Dalela et.al, 2012, Ozcakar et.al, 2011 [36,37]. The frequency of phenotypic ESBL positive *E.coli* 29 (13.49%) isolates were lacked blaTEM, blaCTX-M and blaSHV genes. Which could be false positive results by phenotypic methods or can be possible presence of other ESBL encoding genes such as SFO, BES, BEL, TLA, GES, PER and VEB types and structural changes in penicillin-binding proteins that result in resistance to β-lactam antibiotics [36,38-40].

In this study, the overall prevalence of ESBL genes was 186 (86.51%).Which is similar to other findings reported by Marthie et.al, 2009 in South Africa**,** Ruben et.al, 2014 in Portugal [39,41]. PCR analysis revealed the presence of blaTEM, blaCTX-M and blaSHV genes in ESBL producing *E.coli* was 118(63.4%),68(36.6%) and 0(0) respectively. In the present study, blaTEM was the most predominant genotype of ESBL among *E.coli* isolates. This study is well supported by Ruben et.al,2014 in Portugal, Noha et.al,2020 in Upper Egypt, Majid et.al,2017 in Iran, Pandit et.al,2020 in Nepal, Bali et.al,2010 in Turkish. Michael et.al,2018 in Iraq, Jena et.al, 2017 in India, Seyedjavadi et.al,2016 [41-47].

However, several literatures reported the prevalence of balTEM type ESBL producing *E.coli* was less than this study. In Nepal, some study done by Pokhrel et.al, 2014, Lohani et.al, 2019 reported 29.2%, 34.6% blaTEM type ESBL E.coli respectively [48,49].

In recent years,non-TEM and non-SHV plasmid mediated ESBLs mainly the CTX-M gene, a predominant type of ESBL found in many regions of the world, including Asia,South America,Europe and Africa have been reported and it has been explained under the term "CTX-M β-lactamase pandemic" [50-52].

In the present study, the prevalence of blaCTX-M genes were found to be 68 (36.6%) which concurs with various reports demonstrating the extensive worldwide dissemination of balCTX-M genes in ESBL producing *E.coli* isolates [53]. However, another study from Nepal has reported the high prevalence of blaCTX-M genes (95.2%) by Pokhrel et.al 2014 and (100%) by Lohani et.al 2019 and (91.4%) by Parajuli et.al, 2016 [48,49,54]. Similarly, George et.al, 2015 reported high prevalence of CTX-M type *E.coli* (91.8%) in India [55]. Moreover, the lower prevalence of CTX-M gene among ESBL producing *E.coli* does not co-relate with study done by Moses et.al. and Sharma et.al. [56,57] Kiratisin et.al. reported balCTX-M (99.6%)of ESBL producing *E.coli* from Thailand [58].

The differences in frequencies of the prevalence of these genes may be as a result of differences in time by which isolates were collected and differences in volume and type of antibiotic consumption [59].

Furthermore, multiple harboring of genes in a single ESBL producing *E.coli* was also noted. The most common combination gene was blaTEM + blaCTX-M type 39 (20.96%).Our finding is in agreement with the study by Lohani et.al where (21.2%) of balTEM and blaCTX-M genes were reported [49]. Similar report was shown by Majid et.al.2017 [40]. Whereas genotypic combination of blaSHV + balCTX-M,blaSHV + blaTEM and blaSHV +blaCTX-M +blaTEM were not detected.

The presence of multiple genotypes in a single isolate might be the result of complex antibiotic resistance pattern [60]. Our findings does not agree with the study of Kaftandzieva et al, 2012 and Ibrahim et.al, 2015 where they reported blaTEM and bla SHV type (16%) and (2.27%) respectively [61,62]. Similarly, El bouamri et.al,2015 reported CTX-M +TEM and CTX-M +SHV type (6%) and (12%) respectively [63].

Regarding the blaSHVgene, no blaSHV type E.coli was detected in our study. Which is similar to the study in China [64]. However, several findings in Nepal reported the prevalence of blaSHV gene [48,49,54]. But the prevalence of blaSHV gene reported by Varkey et.al,2014 in India was quite high (66%) [65].

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 by all concern authorities. 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.

Declarations\

1. Ethical Approval: Ethical Approval has been attached here with

2. Consent form: Consent form to participate has been attached here with

3. Compating Interests:The authors declare no conflict of interest

4. Consent for publication: Not Applicable

5. Availability of Data and Materials: Not Applicable

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Authors Contributions

Mahesh Chaudhary: Analysis and interpretation of data, drafting article, final approval to be submitted.

Prof.Dr.Indrani Jadhav: Analysis and interpretation of data, drafting article, final approval to be submitted.

Dr.Megha Raj Banjara: Drafting article, final approval to be submitted.

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