bla$_{CTX-M}$ Mediated Extended Spectrum Beta-lactamase Production in *Escherichia coli* from Urine Samples

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ABSTRACT

Objective: To assess the antimicrobial pattern of *Escherichia coli* isolates from urine samples, determine the proportion of multidrug resistant (MDR) and extended spectrum beta-lactamase (ESBL) and *bla*$_{CTX-M}$ gene of *E. coli* from the urine samples.

Methods: A total of 2452 urine samples were collected and processed from April to October 2023 in clinically suspected urinary tract infection (UTI) patients attending B&B Hospital Pvt. Ltd to identify possible bacteria using standard microbiological procedures. *E coli* isolates were further subjected to antimicrobial susceptibility testing using Kirby Bauer disk diffusion method. Combination disk method was used to identify the ESBL phenotypes. Molecular detection of *bla*$_{CTX-M}$ gene was performed using conventional polymerase chain reaction.

Results: A total of 338 (13.7%) samples showed significant bacterial growth. Maximum bacterial growth was found in outpatients (70.4%) and in patients above 60 years of age. Gram negative bacteria (87.8%) were predominant and *E. coli* (54.4%) was the most frequent. Nitrofurantoin, Aminoglycosides, Carbapenems and Polymixins were effective antibiotics. Among 184 *E. coli* isolates, 54 (29%) were MDR, 30 (16.3%) were ESBL producer. *bla*$_{CTX-M}$ gene was detected in 90% of the phenotypically confirmed ESBL producers.

Conclusion: *E. coli* is the major cause of UTI and *bla*$_{CTX-M}$ is a prime contributor of ESBL production. High rate of resistance to third-generation Cephalosporins, Flouroquinolones and Cotrimoxazole make these antibiotics unsuitable for the treatment of UTI.

Keywords: *Escherichia coli*, Urine, MDR, ESBL, *bla*$_{CTX-M}$

INTRODUCTION

Urinary tract infection (UTI) can be complicated or uncomplicated and caused by both bacteria and fungi (Amna et al 2012). *Escherichia coli* is found to cause most cases of bacterial UTI (Mirzarazi et al 2013). Uropathogenic *E. coli* (UPEC) establish infection due to various factors and has capacity to resist antibiotics (Karam et al 2018).

The production of β-lactamases is the prime contributor of antibiotic resistance and ESBLs are produced by *E. coli* and *Klebsiella pneumoniae* and other bacteria (Guragain et al 2019). Common types of ESBL are SHV, TEM, CTX-M, and among them CTX-M type confer higher resistance to cephalosporins (Paterson and Bonomo 2005). ESBL genes are largely found in plasmid, ESBL production can render Aminoglycosides, Fluoroquinolones and Trimethoprim-Sulfamethoxazole ineffective creating less options for the treatment of ESBL producing bacteria (Gajamer et al 2020).

Antimicrobial resistance (AMR) is emerging threat and ESBL producing genes has stood as a major part of this threat. Limited data are available in surveillance of ESBL producing uropathogenic bacteria and the gene contributing to it. The results of this study can provide information to institute appropriate antibiotics for the treatment of ESBL producing bacteria and

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minimization of further spread of AMR.

MATERIALS AND METHODS

Study site and study period

This was a hospital-based cross-sectional study conducted from April to October 2023 collecting urine samples from clinically suspected urinary tract infected patients. Samples were collected and phenotypically processed at the Department of Microbiology of B&B Hospital Pvt. Ltd., Gwarko, Lalitpur and detection of bla_{CTX-M} gene in *E. coli* was carried out at Central Department of Microbiology, Kirtipur, Kathmandu. Ethical approval of the study was obtained from Institutional Review Committee of Institute of Science and Technology, Tribhuvan University.

Sample size

A total of 2452 mid-stream urine samples following case definition of suspected UTI were included in the study. The required sample size was 89 mid-stream urine samples for the detection of bla_{CTX-M} gene, however, only 30 urine samples which were confirmed ESBL positive were selected.

Culture, isolation and identification of bacteria

Semi-quantitative method using 4mm diameter inoculating loop was used to inoculate the urine samples on Cysteine Lactose Electron Deficient (CLED) agar plates and incubated at 37°C for 24 hours. If no significant growth was observed, it was reported as growth negative. The number of colony forming unit (CFU) per ml of urine was calculated and interpreted. The isolated colony from the plates showing significant bacterial growth were characterized on the basis of morphological characteristics of colony, staining reactions and biochemical reactions (Cheesbrough 2006).

Antibiotic susceptibility test (AST) and detection of ESBLs

Antibiotic susceptibility testing of the isolated *E. coli* was performed by Modified Kirby Bauer Disc Diffusion Method on MHA following Clinical and Laboratory Standards Institute (CLSI) guideline (2020). The antibiotics used were Amikacin (AK 30µg), Ceftriaxone (CTR 30µg), Cefotaxime (CTX 30µg), Ceftazidime (CAZ 30µg), Ciprofloxacin (CIP 5µg), Ofloxacin (OF 5µg), Gentamycin (GEN 10µg), Cotrimoxazole (COT 25µg), Imipenem (IMP 10µg), Meropenem (MRP 10µg), Nitrofurantoin (NIT 300µg), Polymixin B (PB 300U) and Colistin sulphate (CL 10µg). MDR isolates were identified according to Magiorakos et al (2012) stating that isolates when they resist at least one antibiotic in at least three classes of antibiotics.

For screening the ESBL production in *E coli*, Ceftazidime (30 µg) and Cefotaxime (30 µg) discs were used. If the zone of inhibition was ≤ 22 mm for Ceftazidime or ≤ 27 mm for Cefotaxime, the isolates were considered as potential ESBL producers. For confirming the ESBL production, Ceftazidime (30 µg) disk and/or Cefotaxime disk (30 µg) paired with their Clavulanate combination was used. If the zone of inhibition was ≥ 5 mm greater with the combination disk than the Cephalosporin disks alone in any of these two combinations, the *E. coli* isolates were confirmed as ESBL producers (CLSI 2020).

DNA extraction and molecular detection of CTX-M gene

Boiling method was used for extraction of DNA. Briefly, a single bacterial colony from overnight culture was suspended in 100 µL of 50 mM concentration of NaOH and kept in a water bath at 97°C for 3 minutes. It was then immediately subjected to refrigeration at 4°C for 5 minutes. After refrigeration, 16 µL of 1M of Tris-HCl was added and centrifugation was done at 8000 rpm for 2 minutes. The supernatant was collected and stored at -20°C (Gopal et al 2016).

Conventional PCR technique was used to amplify CTX-M gene using primers F: 5′ - TTTGCGATGTGCAGTACCAGTAA-3′; R: 5′ - CTCCGCCTGCCGGTTTTAT-3′ (Nayaju et al 2021). The master mix containing 200 μM of dNTPs, 0.5 U/μL of Taq polymerase in 1X PCR buffer, and 25 mM $MgCl_2$ was used. The PCR was performed in a final volume of 25 µl mixing the 13 µL of the master mix, 8 µL of the double-distilled water, 0.5 µL each of the forward and reverse primer and 3 µL of the template DNA. PCR was done by initial denaturation at 95°C for 15 minutes; followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 3 minutes and a final extension at 72°C for 10 minutes. The PCR products were then analyzed on 2% agarose gel electrophoresis (80V, 60 minutes) with 0.2 μg/mL concentration of ethidium bromide and then visualized by UV-transilluminator (Nayaju et al 2021).

Data analysis

Data were entered using Statistical Package for Social Sciences (SPSS) and analyzed. Chi square test was used

for inferential analysis.

RESULTS

Growth pattern of bacteria in urine samples

Among 2452 midstream urine samples, 338 showed significant bacterial growth belonging to 10 different genera. Among the isolates, Gram negative bacteria (87.8%) were predominating isolates. Of the total isolates, *E. coli* (54.4%) were most frequently isolated bacteria (Table 1).

Table 1: Growth pattern of bacteria in urine samples

Bacterial isolates	Number	Percent
A. baumannii	6	0.2
P. aeruginosa	17	0.7
C. freundii	1	0.03
C. koseri	2	0.1
E. cloacae	1	0.03
K. oxytoca	30	1.2
K. pneumoniae	53	2.2
E. coli	184	7.5
P. mirabilis	2	0.1
M. morganii	1	0.03
E. fecalis	6	0.2
S. aureus	2	0.1
S. saprophyticus	2	0.1
S. epidermidis	19	0.8
Other CONS	12	0.5
No significant growth	2114	86.2
Total	**2452**	**100**

Antibiotic susceptibility pattern of *E. coli*

Among the antibiotics used, the isolates were found to be most sensitive to Colistin sulphate and Polymixin B (98.4%). Antibiotic resistance was found to be highest against Cefotaxime and Ceftriaxone (45.7%) (Table 2).

Table 2: Antibiotic susceptibility pattern of *E. coli* (n=184)

Antibiotic class	Antibiotics	Sensitive No. (%)	Intermediate No. (%)	Resistant No. (%)
Aminoglycosides	Amikacin	166 (90.2)	9 (4.9)	9 (4.9)
	Gentamycin	155 (84.2)	5 (2.7)	24 (13)
Cephalosporin	Ceftriaxone	98 (53.3)	2 (1.1)	84 (45.7)
	Cefotaxime	98 (53.3)	2 (1.1)	84 (45.7)
	Ceftazidime	99 (53.8)	4 (2.2)	81 (44)
Fluoroquinolone	Ciprofloxacin	105 (57.1)	2 (1.1)	77 (41.8)
	Ofloxacin	106 (57.6)	3 (1.6)	75 (40.8)
Sulphonamide and trimethoprim	Cotrimoxazole	108 (58.7)	3 (1.6)	73 (39.7)
Carbapenems	Imipenem	173 (94)	1 (0.5)	10 (5.4)
	Meropenem	173 (94)	1 (0.5)	10 (5.4)
Nucleic acid synthesis inhibitor	Nitrofurantoin	174 (94.6)	5 (2.7)	5 (2.7)
Polymixin	Polymixin B	181 (98.4)	2 (1.1)	1 (0.5)
	Colistin sulphate	181 (98.4)	2 (1.1)	1 (0.5)

Distribution of multidrug resistant and ESBL *E. coli*

Among the 184 *E. coli* isolates, 29% were MDR. For the detection of ESBL producer 51.6% were ESBL screening test positive and only 16.3% were confirmed as ESBL producers (Table 3).

Table 3: MDR and ESBL E. coli in urine samples

Particulars	Number (%) (n=184)
Multidrug drug resistant	54 (29.0)
ESBL screened positive	95 (51.6)
ESBL confirmed isolates	30 (31.4)

Of the total 54 MDR isolates, 25.9% were found to be ESBL producer and of 130 non- MDR isolates, 12.3% were found to be ESBL producers and significant association was found between ESBL production and multidrug resistance (p=0.023) (Table 4).

Table 4: MDR and ESBL E. coli isolates

ESBL/MDR status	ESBL (%)	ESBL non-producer (%)	p value
MDR	14 (25.9)	40 (74.1)	0.023
Non- MDR	16 (12.3)	114 (87.7)	

Distribution of CTX-M gene in phenotypically ESBL positive isolates

It was found that 27 (90%) were found to be CTX-M gene positive (Figure 1).

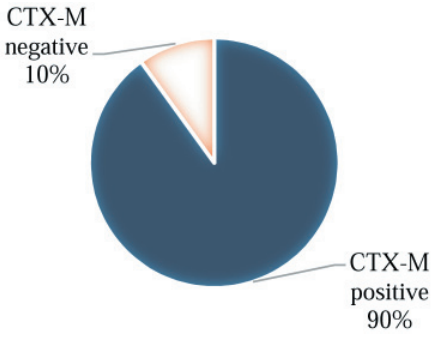

Figure 1: Distribution of CTX-M gene in ESBL positive E. coli isolates

Antibiotic resistance pattern of ESBL positive and negative *E. coli*

Among the ESBL producers, highest (30, 100%) number of isolates were resistant to Ceftriaxone and Cefotaxime followed by Ceftazidime (29, 96.7%) and least (0, 0%) number of isolates to Imipenem and Meropenem. There was significant association (p<0.001) between resistance to Ceftriaxone, Cefotaxime and Ceftazidime and ESBL production and significant association (p=0.044) was also found between ESBL production and resistance to Meropenem and Imipenem (Table 5).

Table 5: Antibiotic resistance pattern of ESBL producing and non- producing E. coli isolates

Antibiotic class	Antibiotics	ESBL producer No. (%)	ESBL non-producer No. (%)	p-value
Aminoglycosides	Amikacin	1 (3.3)	17(11)	0.146
	Gentamycin	3 (10)	26 (16.9)	0.321
Cephalosporin	Ceftriaxone	30 (100)	56 (36.4)	<0.001
	Cefotaxime	30 (100)	56 (36.4)	<0.001
	Ceftazidime	29 (96.7)	56 (36.4)	<0.001
Fluoroquinolone	Ciprofloxacin	18 (60)	61 (39.6)	0.039
	Ofloxacin	17 (56.7)	61 (39.6)	0.084
Sulphonamide and trimethoprim	Cotrimoxazole	16 (53.3)	60 (39)	0.144
Carbapenems	Imipenem	0 (0)	11 (7.1)	0.044
	Meropenem	0 (0)	11 (7.1)	0.044
Nucleic acid synthesis inhibitor	Nitrofurantoin	1 (3.3)	9 (5.8)	0.556
Polymixin	Polymixin B	1 (3.3)	2 (1.3)	0.466
	Colistin sulphate	1 (3.3)	2 (1.3)	0.466

DISCUSSION

In this study, *E. coli* was found to be the leading causative agent of UTI. The prevalence of MDR and ESBL *E. coli* was 29% and 16.3% respectively. bla_{CTX-M} gene was detected in 90% of the phenotypically confirmed ESBL producers.

In this study, we found about one tenth of the urine samples from suspected UTI patients were positive, where Gram-negative bacteria were found to be predominant. It means that Gram- negative bacteria remain a major cause of UTI since a very long time (Joshi et al 2018). *E. coli* was found to be the major etiology constituting more than half percentage as a causative agent. Maintenance of proper sanitation

and health hygiene can help to prevent incidence of UTI in individuals, female and pediatric population in particular.

In this study, around half of the *E. coli* isolates were resistant to all the Cephalosporins used (Ceftriaxone, Ceftazidime and Cefotaxime) and isolates were comparatively less susceptible to Cephalosporins than to other antibiotics. It is often seen that most individuals suffering from simple fever are prescribed Cephalosporins like Ceftriaxone without proper susceptibility testing. This misuse of drug have led to Cephalosporin resistant bacteria (Acharya et al 2011). About 40 percent of the *E. coli* isolates were resistant to the fluoroquinolones, (Ciprofloxacin and Ofloxacin) and Cotrimoxazole. The resistance to Fluoroquinolones was seen similar to studies conducted by Rimal et al (2017) and Khadri and Alzohairy (2010). The resistance shown by isolates towards these drugs could be because of the overuse of these antibiotics because they are cheaply available and are easy to use which necessitates restricting over the counter sales of antibiotics. Around four fifth of the *E. coli* isolates were found to be susceptible to Gentamycin. Similar result was seen another study (Rimal et al 2017). In our study, more than nine tenth of the *E. coli* isolates were susceptible to Amikacin, Nitrofurantoin, Carbapenems and Polymixins. Nitrofurantoin remains the most preferred prescribed oral drug because they are used only for UTIs, have limited systemic absorption and bacteria needs to mutate many times to gain resistance against Nitrofurantoin (Nicolle et al 2006).

In our study, multidrug resistance was found more than one quarter. The rates of multidrug resistance among *E. coli in Nepal* ranges from 38.2 to 95.52% as per different studies (Baral et al 2012; Yadav et al 2015). A study by Shakya et al (2021) showed high rates of MDR in inpatients. However in our study, outpatients exceeded number of inpatients. This could be why multidrug resistance was observed to be comparatively low in our study. It means the antibiotic practices in inpatients or unsuccessful empirical therapy among outpatients who might have ended up as inpatients could be a reason for multidrug resistance.

We found that less than one fifth of *E. coli* isolates were ESBL producers. It means that prevalence of ESBL producing *E. coli* is declining which might be because of presence of ampC β-lactamase which may give false negative tests in the detection of ESBL (Dalela et al 2012). Similar findings were observed in the studies by Chander and Shrestha (2013) and Mahaseth et al (2019) but some studies showed comparatively higher rates of ESBL producing *E coli* (Guragain et al 2019; Nayaju et al 2021).

We found that nine tenth $bla_{CTX\text{-}M}$ gene of the confirmed ESBL *E. coli* isolates possessed ESBL genes. It means that $bla_{CTX\text{-}M}$ gene is disseminating rapidly because this gene has become associated with a variety of mobile genetic elements that contributes to fast bacterial dissemination (D' Andrea et al 2013). Similar prevalence rate of $bla_{CTX\text{-}M}$ gene was seen in other studies (Abdi et al 2014: Nayaju et al 2021). However another study showed lower prevalence of $bla_{CTX\text{-}M}$ gene (Pandit et al 2020; Tabar et al 2016). In our study higher prevalence of $bla_{CTX\text{-}M}$ gene might have been evident because total DNA was extracted by boiling method by which both chromosomal and plasmid mediated $bla_{CTX\text{-}M}$ gene were detected (Gajamer et al 2020).

Our study focused on detection of $bla_{CTX\text{-}M}$ from limited phenotypically positive ESBL *E.coli* from urine samples. Anyway it provides the inference of $bla_{CTX\text{-}M}$ in ESBL production in *E.coli* rendering resistance to beta-lactam antibiotics.

CONCLUSIONS

E. coli is the major cause of UTI. The prevalence of MDR *E. coli* was 29% and of ESBL was 16.3%. $bla_{CTX\text{-}M}$ gene is a major contributor of ESBL in *E. coli*. Early detection of MDR and ESBL should be encouraged which can help in prescribing appropriate antibiotics.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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