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Research Article

Extended spectrum beta-lactamase production and blaCTX-M gene in *Escherichia coli* and *Klebsiella pneumoniae* causing urinary tract infection at a tertiary care hospital in Nepal

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Abstract

Background and objective: Urinary tract infections (UTIs) are the most common bacterial infections where *Escherichia coli (E. coli)* and *Klebsiella pneumoniae* (*K. pneumoniae*) are the predominating pathogens. These pathogens have a high rate of antibiotic resistance and exhibit the production of extended-spectrum beta-lactamase (ESBL). This study investigated the antibiotic resistance pattern and ESBL production of *E. coli* and *K. pneumoniae* isolated from patients with UTIs attending a tertiary care hospital in Nepal by both phenotypic and genotypic techniques.

Materials and methods: A cross-sectional study was performed where 4664 mid-stream urine specimens from suspected UTI cases were cultured. Isolated *E. coli* and *K. pneumoniae* were subjected to antibiotic susceptibility testing by Kirby Bauer disc diffusion method. Genotypic detection of blaCTX-M gene was performed using polymerase chain reaction (PCR).

Results: Out of 4664 urine samples processed, 564 (12.1%) were positive for *E. coli* (475, 10.2%) and *K. pneumonia* (89, 1.9%). Out of the total 564 studied samples, 267 (47.3%) were MDR isolates (*E. coli*: 222, 46.7*%; K. pneumoniae*: 45, 50.6%) and 96 (17%) were positive for ESBL by double disc confirmatory test. Out of 24 ESBL positive *E. coli* and 6 *K. pneumoniae*, 19 (79.2%) and 3 (50%) respectively were positive for blaCTX-M gene.

Conclusion: This study indicates high prevalence of MDR and ESBL producing *E. coli* and *K. pneumoniae* causing UTIs at an urban hospital setting in Nepal.

Introduction

Urinary tract infection (UTI) is a common bacterial infection encountered in medical practice [1-5]. *Escherichia coli* and *Klebsiella pneumoniae* are the two main bacteria frequently linked to urinary tract infections [3,6]. Additionally, these bacteria are also responsible for bloodstream, wound, and respiratory tract infections [7-11]. Antibiotics such as carbapenems, fluoroquinolones, β-lactams, and β-lactam/β-lactamase inhibitors are commonly used to treat urinary tract infections [8,12]. But as of late, many uropathogens have developed resistance to these widely used antimicrobial agents [8,12].

One of the significant classes of β-lactamases known as extended-spectrum β-lactamases (ESBLs)

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is capable of conferring resistance to a wide range of β-lactam antibiotics. These include the extended spectrum (or third generation) cephalosporins (namely cefotaxime, ceftriaxone, and ceftazidime) and monobactams (aztreonam), but not the cephamycins (namely cefoxitin) and carbapenems (imipenem, meropenem, and etrapenem) [2,13-18]. However, β-lactamase inhibitors such as tazobactam, clavulanic acid, and sulbactam can block these enzymes [14,18-21]. Long-term antibiotic exposure, extended hospital stay, instrumentation or catheterization, are the major risk factors for colonization or infection with ESBL-producing organisms [2,6,7,10,14,16,22,23].

Temoniera (TEM), sulfhydryl reagent variable (SHV), and cefotaximase-Munich (CTX-M) enzymes are the sources of the majority of ESBLs, which are encoded by the blaTEM, blaSHV, and blaCTX-M genes respectively [16,22,24,25]. Recently, CTX-M-type beta-lactamases are reported as the most common resistance factors in clinical settings worldwide [26]. Bacteria that possess the blaCTX-M gene are resistant to a wide range of cephalosporin classes. Therefore, it is important to continuously monitor ESBL producing *E. coli* and *K. pneumoniae* causing different types of infections in hospitals and a locality. This study aimed to determine antibiotic resistance pattern, ESBLs production, and blaCTX-M gene in *E. coli* and *K. pneumoniae* isolates from urine samples of suspected UTI cases.

Materials and methods

This was a cross-sectional study conducted in the Department of Microbiology at Alka Hospital, Lalitpur, Nepal, from March 2023 to May 2023. The study population comprised of patients with clinically suspected UTIs from all age groups. The study was approved by Institutional Review Committee – Nobel College with the Ref No: BMM IRC 289/2019.

Information on patient demographics (age, sex, and occupation) and relevant clinical history was collected from patients' records in hospital folders. Mid-stream urine (MSU) sample was collected in a leak-proof, sterile, screw-capped container. Samples held for more than two hours at room temperature and improperly or unlabeled samples, were excluded from the study.

Isolation and Identification of organisms: Urine samples were cultured following standard microbiological guidelines as described elsewhere [27]. Using a sterile calibrated loop (2 mm), the urine samples were streaked directly on MacConkey agar and Blood agar plates. These plates were incubated at 37 °C aerobically and after overnight incubation, they were checked for bacterial growth. Colony count was made, and the positive result was considered for plates showing more than or equal to 10^5 colony-forming units (CFU)/mL of urine based on Kass, Marple, and Sanford criteria [28]. The isolates were identified based on cultural characteristics in MacConkey agar and Blood agar, Gram staining, catalase test, oxidase test, and other relevant biochemical tests as per standard laboratory methods [29].

Antibiotic susceptibility testing: Antimicrobial susceptibility testing (AST) was done by the Kirby-Bauer disk diffusion technique using Muller Hinton agar [30]. All identified isolates of *E. coli* and *K. pneumoniae* were tested for susceptibility against amikacin (30 µg), amoxicillin (10 µg), gentamicin (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cotrimoxazole (25 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), norfloxacin (5 µg), meropenem (10 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 µg), tigecycline (15 µg), polymixin B (10 µg) and colistin (10 µg). Results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) 2016 guidelines [30,31]. The bacterial isolates showing resistance towards three or more different antibiotic classes were considered multidrug-resistant (MDR) [32].

Screening and confirmation of ESBL producers: The screening was done by disc diffusion technique using cefpodoxime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), aztreonam (30 µg) discs. For confirmation, combined disc test was performed using ceftazidime (30 µg) alone, and ceftazidime + clavulanic acid (20 µg + 10 µg). A difference of ≥5 mm between the zone diameters of either of cephalosporin disks and their respective cephalosporin/clavulanate disk was taken to be phenotypic confirmation of ESBL production [30].

*Amplification and detection of bla*CTX-M *gene using PCR method*: From confirmed ESBL producers,

plasmids were extracted using standard alkaline hydrolysis method. These plasmids served as the template for PCR. The *bla*CTX-M gene amplification was performed by PCR technique using specific primer: 5'-TTTGCGATGTGCAGTACCAGTAA-3' as a forward primer and 5'- CTCCGCTGCCGGTTTTATC-3' as a reverse primer [19]. A final volume of 25 µl was prepared by adding 12.5 µl master mix green go-Taq, 1 µl of forward and reverse primer each, 8.5 µl nuclease-free water, and 2 µl bacterial DNA. Amplification was performed with the following cycling conditions: initial denaturation at 94^0C for 5 minutes; followed by 30 cycles each of extended denaturation at 95^0C for 45 seconds, annealing for 62^0C for 45 seconds, extension at 72^0C at 30 seconds, and extended extension at 72^0C for 10 minutes. The PCR products and the DNA marker were visualized by using 2% agarose gel electrophoresis.

Quality Control: *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls, respectively. For PCR already confirmed *E. coli* strains harboring *bla*CTX-M were taken as a positive control and nuclease free water as the negative control.

Statistical analysis: The statistical analysis was performed in Statistical Package for Social Sciences (SPSS) version 25.0. Discrete variables were expressed into percentages. Categorical variables were compared using the Chi square test and a p-value <0.05 was considered a statistically significant finding.

Results

Out of 4664 urine samples processed, 564 (12.1%) were positive for *E. coli* (475, 10.2%) and *K. pneumonia* (89, 1.9%). Among 564 studied samples, the sex wise distribution of the patients showed that 110 (19.5%) isolates were from male and 454 (80.5%) isolates were from female patients. Most organisms were isolated from the age group 21-40 years. Least organisms were isolated from age group 0 -20 years (Table-1).

Out of the total 564 studied samples, 267 (47.3%) were MDR isolates (*E. coli*: 222, 46.7*%; K. pneumoniae*: 45, 50.6%) and 96 (17%) were positive for ESBL by double disc confirmatory test (Table-2). Out of 222 MDR *E. coli*, 160 (72.1%) were ESBL positive by screening test while 82 (36.9%)

Table-1: *Age and sex-wise distribution of cases from whom E. coli and K. pneumoniae were isolated*

Age group (yrs)	Total isolates N	Isolates from		*E. coli*			*K. pneumoniae*		
		Male N	Female N	Male N	Female N	Total N	Male N	Female N	Total N
0-20	56	16	40	13	36	49	3	4	7
21-40	257	10	247	7	210	217	3	37	40
41-60	134	40	94	30	81	111	10	13	23
> 61	117	44	73	32	66	98	12	7	19
Total	564	110	454	82	393	475	28	61	89

Table-2: *ESBL positivity in MDR and non-MDR E. coli and K. pneumoniae*

Organism	Total isolates	Total MDR isolate	Total ESBL positive*	ESBL positive by Screening test	ESBL positive by Double disc test*	Total Non-MDR	ESBL positive by Screening test	ESBL positive by Double disc test*
	N	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
E. coli	475	222 (46.7)	86 (18.1)	160 (72.1)	82 (36.9)	253 (53.3)	21 (8.3)	4 (1.6)
K. pneumoniae	89	45 (50.6)	10 (11.2)	27 (60)	10 (22.2)	44 (49.4)	3 (6.8)	0
Total	564	267 (47.3)	96 (17)	187 (33.2)	92 (16.3)	297 (52.7)	24 (4.3)	4 (0.7)

*Note: *confirmatory test; p > 0.05, compared between E. coli and K. pneumoniae for MDR and ESBL rates.*

Table-3: *ESBL positivity in MDR E. coli and K. pneumoniae isolated from male and female patients*

Gender	*E. coli*				*K. pneumoniae*			
	Total isolated N (%)	ESBL positive by			Total isolated N (%)	ESBL positive by		
		MDR strain N (%)	Screening test N (%)	Double disc test* N (%)		MDR strain N (%)	Screening test N (%)	Double disc test* N (%)
Male	107 (22.52%)	52 (48.6)	46 (88.5)	17 (32.7)	3 (3.4)	3 (100)	3 (100)	1 (33.3)
Female	368 (77.47%)	170 (46.2)	135 (79.4)	69 (40.6)	86 (96.6)	42 (48.8)	27 (64.3)	9 (21.4)
Total	475	222	181	86	89	45	30	10

*Note: p>0.05, compared between male and female for ESBL positivity of E. coli and K. pneumoniae; *confirmatory test*

Table-4: *Age wise distribution of MDR and ESBL producing E. coli and K. pneumoniae*

Age group Yrs	*E. coli*				*K. pneumoniae*			
	Total isolates N (%)	MDR strains N (%)	ESBL positive by		Total isolates N (%)	MDR strains N (%)	ESBL positive by	
			Screening test N (%)	Double disc test* N (%)			Screening test N (%)	Double disc test* N (%)
0-20	49	22 (44.9)	19 (38.8)	9 (18.4)	7 (7.9)	4 (57.1)	2 (28.6)	2 (28.6)
21-40	217	82 (37.8)	61 (28.1)	31 (14.3)	40 (44.9)	16 (40)	10 (25)	4 (10)
41-60	111	55 (49.5)	46 (41.4)	20 (18)	23 (25.8)	12 (52.2)	8 (34.8)	1 (4.3)
> 61	98	63 (64.8)	55 (54.9)	26 (28.2)	19 (12.4)	13 (81.8)	10 (54.5)	3 (27.3)
Total	475	222 (46.7)	181 (38.1)	86 (18.1)	89 (100)	45 (50.6)	30 (33.7)	10 (11.2)

Note: p<0.05, compared between age group > 61 years and other groups; p <0.05, compared 41-60 age group with lower age groups (0-20 and 21-40 yrs groups).

became ESBL positive by confirmatory test. Similarly for *K. pneumoniae*, the ESBL positivity rate by screening and confirmatory test was 60% and 22.2% respectively. The rate of ESBL positivity among non-MDR isolates of *E. coli* was 1.6% while none of the non-MDR *K. pneumoniae* was positive for ESBL (Table-2). There was a significant association between ESBL production and MDR isolates (p<0.001). But the rate of MDR and ESBL positivity was not significantly (p > 0.05) different between *E. coli* and *K. pneumoniae*.

Detail rate of ESBL positivity in MDR *E. coli* and *K. pneumoniae* isolated from male and female patients is shown in Table-3. Among 222 MDR *E. coli* isolates, 52 (48.6%) and 170 (46.2%) were from male and female patients respectively. Out of 107 and 368 *E. coli* isolates from male and female patients, 52 (48.6%) and 170 (46.2%) isolates were MDR respectively. The rate of ESBL producing MDR *E. coli* from male and female cases was not significantly (p>0.05) different (32.7% vs. 40.6%). Similarly, the rate of ESBL positive *K. pneumoniae* isolated from male and female patients was not significantly (p>0.5) different (33.3% s. 21.4%).

Age wise distribution of MDR and ESBL producing isolates is shown in Table-4. Rate of MDR E. coli and *K. pneumoniae* was significantly (p<0.05) higher among the older age groups compared to younger groups. The rate of ESBL producing *E. coli* and *K. pneumoniae* was not significantly different among the groups.

About 40.7% to 67.7% *E. coli* isolates were resistant to quinolones, amoxicillin+clavulanate, co-trimoxazole and imipenem (Table-5). Except imipenem, the rate of resistance of *K. pneumoniae*

to these antibiotics was between 50%-90%. None of the *E. coli* and *K. pneumoniae* was resistant to colistin. Amikacin, meropenem, tigecycline, nitrofurantoin, polymixin-B, and colistin were the most effective antibiotic for ESBL positive *E. coli* and *K. pneumoniae*.

Table-5: *Antibiotic susceptibility pattern of ESBL producing E. coli and K. pneumoniae*

Antimicrobial agents	*E. coli*		*K. pneumoniae*	
	ESBL +ve isolate tested Number	Resistant N (%)	ESBL +ve isolate tested Number	Resistant N (%)
Amikacin	86	16 (18.6)	10	3 (30)
Gentamicin	86	21 (24.4)	10	3 (30)
Ciprofloxacin	86	51 (59.3)	10	8 (80)
Norfloxacin	86	44 (51.2)	10	9 (90)
Nalidixic Acid	86	35 (40.7)	10	5 (50)
Amox+ clavulanate	86	57 (66.3)	10	7 (70)
Co-trimoxazole	86	45 (52.3)	10	7 (70)
Nitrofurantoin	86	8 (9.3)	10	4 (40)
Cefop+sulbactam	40	8 (20.0)	5	4 (80)
Pip+tazobactam	34	4 (11.8)	4	1 (25)
Imipenem	31	21 (67.7)	4	0
Meropenem	31	2 (6.5)	4	1 (25)
Tigecycline	23	1 (2.9)	4	0
Polymyxin-B	23	1 (2.9)	4	0
Colistin	23	0	4	0

Note: Amox+Clavulanate: Amoxicillin+clavulanate; Cefop+sulbactam: Cefoperazone+sulbactam; Pip+tazobactam: Piperacillin+tazobactam

Table-6: *Distribution of blaCTX-M gene among ESBL-producing E. coli and K. pneumoniae isolates*

Gender	*E. coli (n=24)*		*K. pneumoniae (n=6)*		Total
	CTX-M positive	CTX-M negative	CTX-M positive	CTX-M negative	
	N (%)	N (%)	N (%)	N (%)	N 9%)
Male	3 (12.5)	0	1 (16.7)	2 (33.3)	6 (20)
Female	16 (66.7)	5 (20.8	2 (33.3)	1 (16.7)	24 (8)
Total	19 (79.2)	5 (20.8)	3 (50)	3 (50)	30 (100)

Discussion

The present study sought to determine the rate of ESBL producing *E. coli* and *K. pneumoniae* isolates causing urinary tract infections. We found that these organisms had a high level of antibiotic resistance and were the most frequent cause of UTIs. In our series, *E. coli* and *K. pneumoniae* was isolated from 12.1% of the 4,664 urine specimens evaluated. This finding is comparable to other reported studies from Nepal [33-35]. Our study sought to determine how common ESBL producing E. *coli* and *K. pneumoniae* isolates from urinary tract infections. Of the total *E. coli* and *K. pneumoniae* isolates, 17% were ESBL producers. Other studies from Nepal and the region reported variable rates of ESBL producing *E. coli* and *K. pneumoniae* in urine and different clinical samples [33-38].

In our study, blaCTX-M gene was present in 79.2% and 50% of *E. coli* and *K. pneumoniae* respectively. Others have reported higher prevalence of blaCTX-M gene from 66.6% to 100% in *E. coli* and *K.*

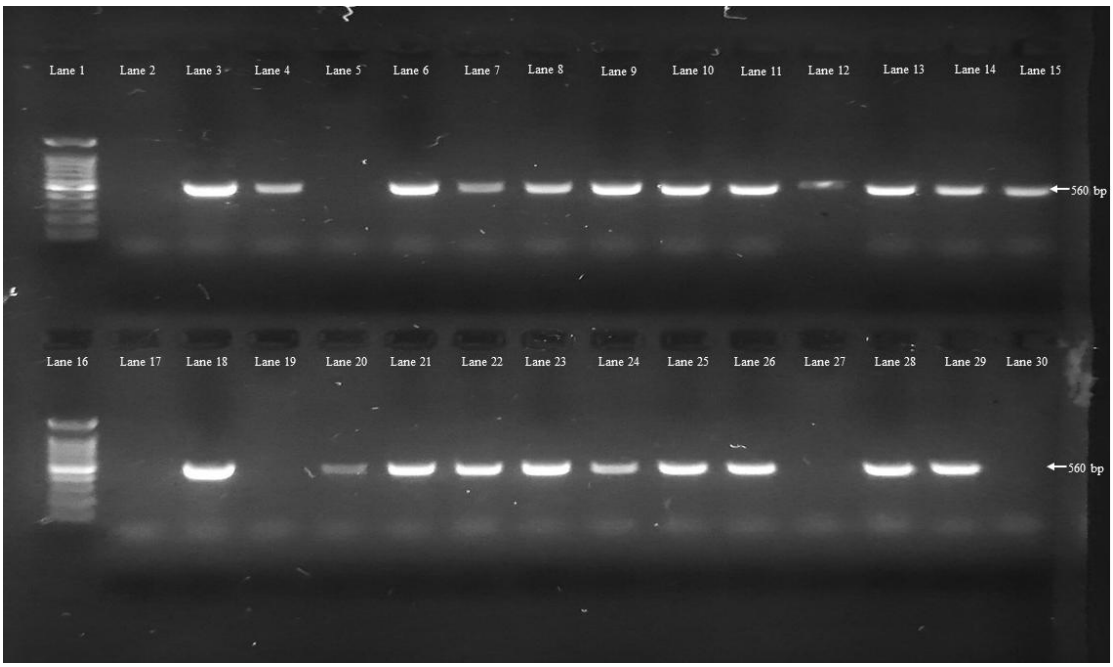

Figure-1*: Agarose gel electrophoresis of the amplified PCR product for blaCTX-M gene. Lane 1 and 16:100 bp DNA markers; Lane 2 and 17: negative control; Lane 3 and 18: positive control; Lane 4 to 15 and Lane 19 to 30: isolates tested for the presence of blaCTX-M gene (560 bp).*

pneumoniae [19,35,39,40]. Variations in the volume and kind of antibiotic use as well as variations in the time of isolate collection may account for the variations in frequencies and prevalence of these genes.

One of the noteworthy findings in the present study among ESBL producers was the high resistance rate to imipenem (67.7%) which is contrary to that found by Shakya et al [38] and Zeynudin et al [41] who reported the imipenem résistance rate as 0% and 1.90%, respectively. High imipenem resistance can be attributed to the prevalence of carbapenemase β-lactamases as well as a rise in the haphazard use of the antibiotics to treat infections [34]. Furthermore, both of the ESBL isolates in our investigation showed high resistance to amoxicillin+clavulanic acid. The finding is consistent with the findings of Shashwati et al [37]. Multidrug resistance trends can differ between countries or even hospitals within the same nation due to differences in antibiotic prescribing practices during infection and lapses in an efficient infection control program.

The emergence of MDR and ESBL producing *E. coli* and *K. pneumoniae* isolates with high antibiotic – resistant rates to commonly used antibiotics and predominance of blaCTX-M-beta lactamase gene poses a serious concern to the clinicians and microbiologists. Regular monitoring of antibiotic susceptibility and associated genes along with rationale use of antibiotics for treating the predominant pathogens like *E. coli* and *K. pneumoniae* in healthcare facilities is essential to contain the spread of antibiotic résistance.

Author Contributions

GP: Conceptualization and designing of the study, approval of SOP for the study, supervised the study, and validating the results, manuscript – writing, editing, reviewing and submitting; AK, PK and CT: Writing SOP for the study, sample collection, performing tests, result analysis, and reporting of the results, manuscript writing, literature search.

Conflict of Interest

The authors have no conflicts of interest to declare.

Ethics approval

The ethical approval was granted by Institutional Review Committee – Nobel College with the Ref No: BMM IRC 289/2019.

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