

Plasmid Profiling and Occurrence of β -Lactamase Enzymes in Multidrug-Resistant Uropathogenic *Escherichia coli* in Kathmandu, Nepal

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Introduction: Extended-spectrum β -lactamases (ESBL) among Gram-negative bacteria, predominantly *Escherichia coli* (*E. coli*), in Nepal, have been rising. The main objectives of this study were to determine the prevalence of uropathogenic *E. coli*, antibiotic resistance, ESBLs, ABLs (AmpC type β -lactamases), MBLs (metallo- β -lactamases) and KPCs (*Klebsiella pneumoniae* carbapenemases) and their correlation with plasmid profiling patterns among patients with urinary tract infections in a tertiary hospital in Kathmandu, Nepal.

Methods: The mid-stream urine samples collected from patients were inoculated in cystine–lactose–electrolyte-deficient (CLED) agar plates. *E. coli* producing ESBLs, ABLs, MBLs/ KPC were identified phenotypically using standard microbiological methods. Plasmids were extracted by alkaline lysis method from *E. coli* isolates and profiled using agarose gel electrophoresis.

Results: Out of the total 2661 urine samples, *E. coli* were isolated in 64.34% (507/788), among which 170 (33.53%) were multidrug-resistant (MDR) isolates. All MDR isolates were resistant to amoxicillin and third-generation cephalosporins but were highly sensitive to imipenem (94.12%, 160/170), amikacin (92.94%, 158/170) and nitrofurantoin (86.47%, 147/170). Among 170 MDR isolates, 78.2% (133/170) were ESBLs, 46.3% (50/170) were AmpC, 11.2% (19/170) were MBL and 0.6% (1/170) were KPC producers. Coproduction of β -lactamases was detected in 24.12% (41/170) of isolates. *E. coli* isolates showed one plasmid (>33.5 kb), which was present in all the isolates. Overall, 44 different plasmid profile groups were identified based on molecular weight and number of plasmids. β -Lactamase producers were relatively resistant to the higher number of antibiotics tested (≤ 10) than non-producers (≤ 8), and the number of plasmids were higher in β -lactamase producers (≤ 7) than those in non-producers (≤ 5).

Conclusion: The higher prevalence of the ESBLs, AmpCs, KPCs and MBLs along with their coproduction in *E. coli* isolates highlights the importance of routine surveillance of ESBLs, AmpCs, KPCs and MBLs in microbiology laboratories using various phenotypic methods.

Keywords: uropathogenic *Escherichia coli*, antibiotic resistance, ESBL, extended-spectrum β -lactamases, AmpC type β -lactamases, MBL, metallo- β -lactamases, KPC, *Klebsiella pneumoniae* carbapenemases

Introduction

Antimicrobial resistance is one of the greatest challenges in modern empirical treatment of infectious diseases.¹ For the treatment of infections, especially caused by Gram-negative bacteria, fluoroquinolones, cephalosporins, β -lactams and β -lactamases

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inhibitors alone or in combination are frequently used.^{2,3} However, resistance to such drugs is the major burden in developing countries.¹ Among them, prevalence of beta-lactamases-producing pathogens is on the rise causing a threat to patients⁴⁻⁶ with spread of CTX-M genes; encoding genes have been seized from the chromosome of *Kluyvera* spp. The *ctx* gene is retained on a bacterial plasmid that is spread among the members of family *Enterobacteriaceae*, particularly in *E. coli*.⁷

The selective pressure induced by continuous and uncontrolled use of β -lactam antibiotics has triggered the emergence of diverse form of mutated novel beta-lactamase enzymes.^{8,9} Increasingly, *E. coli* have emerged as prominent bacteria for producing such newer β -lactamases. They comprise of plasmid-mediated AmpC β -lactamases (eg, *CMY* types), extended-spectrum β -lactamases (ESBLs, eg, *TEM*, *SHV*, *CTX-M* types), and carbapenemases (*Klebsiella pneumoniae* carbapenemase (KPC) types, metallo- β -lactamases (MBLs) and OXA types).^{10,11} *CMY*, *CTX-M*, and *NDM* types of β -lactamases in *E. coli* are predominantly responsible for the growing resistance to β -lactam antibiotics.¹²

Evidence of integron mediated resistant is sufficient in bacterium. These genes are usually found on plasmids encoding resistance to aminoglycosides, sulfonamides, tetracyclines and other antibiotics.¹³ Those carrying R-plasmids cause serious problems among antibiotic-resistant bacteria because of their propensity to spread rapidly.¹⁴ A laboratory evidence of horizontal transfer of multi-antibiotic-resistant plasmids among clinical isolates indicates further spread.¹⁵

Antibiotic-resistant genes in Gram-negative bacteria are located on the plasmids, which are responsible for resistance to a number of antimicrobial agents.¹⁶ Assigned plasmids are self-ruling DNA molecules equipped for self-transmission between cells, including their capacity to activate a part of the chromosome through high-recurrence recombination.¹⁷ The ability to acquire novel genes by plasmids through mobile genetic elements such as transposons or insertion sequences, and their propensity to replicate in a wide range of hosts, makes them perfect vectors for the spread of AMR.¹⁸ Identifying the characteristics of plasmids and their behavior in different bacterial hosts provides significant knowledge regarding the transmission of AMR. Molecular characterization of plasmid and genotypes can help to understand whether the spread of AMR genes is caused by epidemic plasmids in different hosts or by clonal spread of bacterial organisms. Plasmids are important for horizontal gene transfer in a close family of bacteria. These are responsible for the

spread of antibiotic resistance genes in the environment. Thus, plasmid profiling helps to identify the potential of the spread of resistant genes. Plasmid profiles will be useful in the surveillance of outbreaks and in tracing antibiotics resistance.¹⁹

UTI is very common and can affect patients well beyond reproductive age.²⁰ Although recognized as a most prevalent illness in infectious diseases wards in the hospitals, routine laboratory diagnoses with antibiotic profiles and susceptibility tests are rarely done.^{4,20-22} In most cases, only basic routine urine microbiological tests are conducted before prescribing the empirical treatment, and this may be even more prominent in non-tertiary hospitals and peripheral health facilities.⁴

Prescribing antibiotics without routinely identifying the organisms and their antimicrobial susceptibility can contribute to antibiotic resistance including ESBLs-producing organisms.²³ Additionally, in Nepal, antibiotics are available over the counter (without a prescription) from drug shops and dispensers.²⁴ High empirical treatment and unnecessary use of antibiotics in viral infections can further facilitate the development of AMR.⁴ This is a major problem and can increase the ESBLs-, AmpCs-, KPCs-, MBLs-mediated resistance.

Several studies in the past have investigated the prevalence of pathogens producing ESBLs among inpatients. Studies have shown the varying prevalence of ESBLs producers, for instance, 27.7% in Pokhara,²³ 55% in Kathmandu,²⁵ 64% in Chitwan,²⁶ and 43% in Children Hospital in Kathmandu in 2018.²⁷ Another study reported 35.9% of ESBLs-producing *E. coli* isolates among outpatients at a tertiary care hospital in Kathmandu.²⁸ However, a study from Lalitpur district reported 6.8% of ESBLs-producing isolates.²⁹ Studies have shown the wide range in prevalence of ESBLs producers (10% to 64%) in different hospitals/settings from various samples. However, there are very few studies that have explored the organisms producing ESBLs amongst the uropathogens from the clinical samples of patients suspected or diagnosed with urinary tract infections. In addition, studies so far have used only phenotypical methods to detect and determine the prevalence of ESBL organisms. It is established that ESBLs and AmpC are the main mechanisms of resistance to β -lactam antibiotics, but the genotypic mechanism of resistance varies globally. Therefore, providing the prevalence of circulating β -lactamase genes in each geographic region can be important. Furthermore, multidrug resistance in bacteria may result from the

accumulation of multiple genes by resistance plasmids (R-Plasmids) and/or by the increased expression of genes that code for multidrug efflux pumps.³⁰

The main objectives of this study were to determine the prevalence of *E. coli* producing ESBLs, AmpC (AmpC type β -lactamases), MBL (metallo- β -lactamase) and KPC (*Klebsiella pneumoniae* carbapenemases), and their correlation with plasmid profiling (genotyping) patterns among the patients suspected of or with urinary tract infection in a tertiary hospital in Kathmandu, Nepal.

Methods

A hospital-based cross-sectional study was conducted from October 2013 to March 2014 at Kathmandu Model Hospital, Kathmandu, Nepal. A total of 2661 mid-stream urine samples were collected from out-patients department (OPD) and intensive care unit (ICU) during the study period. The samples were collected in a clean, leak-proof container with no visible signs of contamination and were labeled properly with demographic information of patients. The samples from patients with a recent history of antibiotics therapy were excluded from this study. A written informed consent was obtained from the patients for participation and collection of samples. The cultured urine samples were considered significant when the number of colonies were more than 10^5 cfu/mL.

Identification of *E. coli*

Urine samples were cultured on cystine–lactose–electrolyte-deficient (CLED) agar plates by semi-quantitative culture technique.³¹ The isolation and identification of *E. coli* were performed following standard microbiological techniques as described by the American Society of Microbiology (ASM).³²

“Antimicrobial Susceptibility Testing”

“Antimicrobial susceptibility testing” (AST) was performed using modified Kirby–Bauer disk diffusion method following the guidelines of Clinical and Laboratory Standards Institute guidelines (CLSI).³³ Sixteen common antibiotic discs from HiMedia: meropenem (10 μ g), amoxycillin (10 μ g), cefotaxime (30 μ g), cefixime (30 μ g), ceftriaxone (30 μ g), ceftazidime (30 μ g), ceftazidime (30 μ g), cotrimoxazole (25 μ g), nitrofurantoin (300 μ g), levofloxacin (5 μ g), amikacin (30 μ g), gentamicin (10 μ g), cefoperazone/sulbactam (75/30 μ g), piperacillin/tazobactam (100/10 μ g), imipenem (10 μ g), doxycycline (30 μ g) were used. *E. coli* that were resistant to at least one agent in

three or more antibiotic categories were characterized as multi-drug-resistant *E. coli*.³⁴

Screening and Phenotypic Confirmation of *E. coli* Producing ESBLs, AmpCs, MBLs and KPCs

Detection of ESBLs Producers

E. coli producing ESBLs was screened by using cefotaxime (30 μ g), ceftazidime (30 μ g) or ceftriaxone (30 μ g) following CLSI guidelines.³³ ESBLs producers were confirmed by combined disc assay using SD240 Hi-Media ESBL detection kit consisting of ceftazidime (30 μ g) and ceftazidime (30 μ g) plus clavulanic acid (10 μ g).

Detection of AmpCs Production

MDR isolates were screened for AmpCs production using cefoxitin (30 μ g) with screening cutoffs of ≤ 18 (ie, the CLSI susceptible breakpoints)³⁵ and were further subjected to phenotypic confirmation (Combined Disc Assay) using cefoxitin (30 μ g) alone and in combination with cloxacillin (200 μ g). Increment of the zone of inhibition by ≥ 4 mm was considered as positive result.³⁶

Detection of MBLs and KPCs

E. coli isolates were considered as MBLs producers when the inhibitory zone diameters around the meropenem disc with ethylene diamine tetra-acetic acid (EDTA) and the meropenem disc with phenylboronic acid, PBA+EDTA had increased to ≥ 5 mm as compared to the inhibitory zone diameter of meropenem disc alone. Similarly, KPCs producers among *E. coli* isolates were considered when the inhibitory zone diameter around the meropenem disc with PBA and the meropenem disc with PBA+EDTA had increased to ≥ 5 mm as compared to the inhibitory zone diameter of meropenem disc alone.³⁷ The reference strains, ESBLs producing *E. coli* NCTC 13351, non-ESBLs producing *E. coli* ATCC 25922, MBLs producing *E. coli* NCTC 13476 and non-MBLs producing *E. coli* ATCC 25922 from Institute of Medicine, Tribhuvan University Teaching Hospital, were included as controls in the study.

Extraction and Profiling of Plasmid DNA

Plasmid DNA was extracted by alkaline lysis method.³⁸ The extracted DNA was separated using 0.8% agarose gel electrophoresis at 120V for 1 hour, stained with SYBR safe stain solution and a photograph of the stained gel was taken after exposure on UV trans-illuminator. Reference as

super mix plasmid DNA marker (GeNei™, Genei Laboratories Private Limited, India) was used for the estimation of plasmid sizes. Plasmid profiles were created by grouping strains possessing the same number of plasmid bands and molecular size.³⁹

Results

Out of 2661 urine samples, 29.61% (788/2661) were positive for a urinary pathogen. *E. coli* was the predominant bacterial species accounting for 64.34% (507/788) of total isolates in which 170 (33.53%) were multidrug-resistant. All MDR isolates were resistant to amoxicillin and third-generation cephalosporins (ceftriaxone, cefotaxime, cefixime and ceftazidime) but were highly sensitive to imipenem (94.12%, 160/170), amikacin (92.94%, 158/170) and nitrofurantoin (86.47%, 147/170) (Table 1).

Antibiotic-resistant pattern of MDR *E. coli* showed 18 different resistant phenotypes (Table 2). The isolates seemed to be resistant to the number of antimicrobials that ranged from 6 to 16 of the commonly used antibiotics. The resistance pattern to a different class of antibiotics among β -lactamase producer and non-producer MDR isolates of *E. coli* is shown in Table 3.

β -Lactamase production was observed in 162 bacterial isolates, out of which 133 (82.1%) were ESBLs producers, followed by 50 (30.84%) AmpCs producers, 19 (11.72%) MBLs producers and 1 (0.62%) KPCs producers. The co-production of ESBLs and AmpCs was observed in 33 (20.37) isolates, followed by 5 (3.09%) ESBLs and MBLs, 1 (0.62%) MBLs and KPCs and 2 (1.23%) MBLs and AmpCs co-producers (Figure 1).

The β -lactamase producers were relatively resistant to a higher number of antibiotics used (highest: 16 antibiotics and nine different categories) while only a few isolates [1.76% (3/170)] were resistant to non-ESBLs producers to a maximum of 12 antibiotics (only seven different antibiotic categories) in this study. Seven plasmids were found in β -lactamase producers and a maximum of 5 plasmids were found in non-ESBLs producers (Table 3). *E. coli* isolates on plasmid profiling showed that one plasmid of greater than 33.5 kb size was present in all the isolates. The number of plasmids in the isolates varied from 1 to 7 along with the size ranging from 0.8 kb to >33.5 kb. Forty-four different plasmid profile patterns were obtained based on molecular weight and number of plasmids content. The number of isolates per plasmid profile group varied from 1 to 20. The maximum number of isolates had two plasmids [67 (39.41%)], followed by three plasmids [29 (17.06%)],

and one plasmid [20 (11.76%)]. The most frequent molecular weight pattern obtained among 20 (11.76%) isolates was >33.5 kb, followed by 19 (11.18%) isolates possessing two plasmids of 33.5kb and >33.5kb sizes (Table 4).

Non- β -lactamase-producing isolates were found to possess one, two and five plasmids, whereas β -lactamase producers had multiple plasmids. Most of the uropathogenic *E. coli* producing ESBLs, AmpC and ESBLs/AmpCs had a variable number of plasmids with one plasmid size (>33.5 kb) in common (Table 5). MBLs producers and co-producers of MBLs with AmpCs, and ESBLs and KPCs had few number of plasmids; however, all of them contained plasmid size greater or equal to 33.5 kb in common. The lower molecular size plasmids showed no significant role in β -lactamases production. The lowest molecular weight plasmid, ie, 0.8 kb, was found in non β -lactamase-producing isolates. Plasmid DNA of clinical isolates of MDR *E. coli* was separated by molecular weight on agarose gel stained with ethidium bromide (Figure 2).

Discussion

E. coli is a predominant cause of both community and nosocomial urinary tract infections.⁴⁰ The trend of emerging multiple antibiotic-resistant *E. coli* among clinical samples raises a major concern for the clinician. The findings of this study show a high level of resistance among *E. coli* against antibiotics with sensitivity to imipenem (94.12%), amikacin (92.94%) and nitrofurantoin (86.47%). Consistent results of resistance have been reported from Saudi Arabia,⁴¹ Egypt⁴² and India.⁴³ *E. coli* isolates from clinical settings showed resistance to nitrofurantoin that ranged from 2.4% to 6.5% and resistance to amikacin (2%) while resistance to imipenem was only 0.3%.⁴¹ Our findings are consistent with the study from India which showed least resistance to amikacin (13.9%) and imipenem (2.3%).⁴³ Many studies have shown that meropenem has greater potency than imipenem by 4–16 folds even in *E. coli* and other members of *Enterobacteriaceae*.^{44,45} However, contrastingly, this study found a higher sensitivity of imipenem (94.12%) than meropenem (12.35%). One study from India has shown consistent findings with our study.⁴⁶ The differences in resistance profile with the other studies can result from differences in infection epidemiology, prescription patterns, and socio-demographic features of the population. In this study, we included only patients who presented with suspected UTI in contrast to other studies

Table 1 Antibiotic Susceptibility Pattern of MDR *E. coli* Isolates (n=170)

| Mode of Action | Antimicrobial Category | Antimicrobial Agents | Sensitive N (%) | Resistant N (%) |
|-----------------------------------|-----------------------------------|-------------------------|-----------------|-----------------|
| Cell wall synthesis inhibitors | Aminopenicillin | Amoxycillin | 0 | 170 (100) |
| | | Cefixime | 0 | 170 (100) |
| | | Cefotaxime | 0 | 170 (100) |
| | | Ceftriaxone | 0 | 170 (100) |
| | | Cefoperazone/Sulbactam | 91 (53.53) | 79 (46.47) |
| | | Ceftazidime | 0 | 170 (100) |
| | Carbapenems | Imipenem | 160 (94.12) | 10 (5.88) |
| | | Meropenem | 21 (12.35) | 149 (87.65) |
| | β-Lactamase inhibitors | Piperacillin/Tazobactam | 108(63.53) | 62 (36.47) |
| Protein synthesis inhibitors | Aminoglycosides | Amikacin | 158 (92.94) | 12 (7.05) |
| | | Gentamicin | 146 (85.88) | 24 (14.12) |
| | Tetracyclines | Doxycycline | 61 (35.88) | 109 (64.12) |
| Nucleic acid synthesis inhibitors | Fluoroquinolones | Ciprofloxacin | 7 (4.12) | 163 (95.88) |
| | | Levofloxacin | 24 (14.12) | 146 (85.88) |
| | Nitrofurans | Nitrofurantoin | 147 (86.47) | 23 (13.53) |
| Folate pathway inhibitors | Trimethoprim and sulfamethoxazole | Cotrimoxazole | 24 (14.12) | 146 (85.88) |

Table 2 Antibiotic Resistance Pattern of Clinical Isolates of MDR *E. coli* Isolates

| Resistance Phenotype (RP) | Resistance Pattern | No. of Isolates Showing the Pattern % (n=170) |
|---------------------------|---|---|
| RP-1 | AmxCtxCtrCfmCazCipLevCotCfsPitMrpDox | 26 (15.29%) |
| RP-2 | AmxCtxCtrCfmCazCipLevCotMrpDox | 25 (14.71%) |
| RP-3 | AmxCtxCtrCfmCazCipLevCotNitCfsPitMrpDox | 14 (8.24%) |
| RP-4 | AmxCtxCtrCfmCazCipLevCotMrp | 14 (8.24%) |
| RP-5 | AmxCtxCtrCfmCazCipLevCotDox | 11 (6.47%) |
| RP-6 | AmxCtxCtrCfmCazCipLevMrp | 10 (5.88%) |
| RP-7 | AmxCtxCtrCfmCazCipLevCotCfsMrp | 9 (5.29%) |
| RP-8 | AmxCtxCtrCfmCazCipMrp | 8 (4.71%) |
| RP-9 | AmxCtxCtrCfmCazCipLevCotGenCfsMrpDox | 7 (4.12%) |
| RP-10 | AmxCtxCtrCfmCazCot | 7 (4.12%) |
| RP-11 | AmxCtxCtrCfmCazCipLevCotAkGenCfsPitMrplpmDox | 7 (4.12%) |
| RP-12 | AmxCtxCtrCfmCazCipCotMrpDox | 6 (3.53%) |
| RP-13 | AmxCtxCtrCfmCazCipLevCfsPitMrpDox | 6 (3.53%) |
| RP-14 | AmxCtxCtrCfmCazCipLevCotNitMrp | 5 (2.94%) |
| RP-15 | AmxCtxCtrCfmCazCipCot | 4 (2.35%) |
| RP-16 | AmxCtxCtrCfmCazCipLevCotGenMrp | 4 (2.35%) |
| RP-17 | AmxCtxCtrCfmCazCipLevCotAkGenCfsPitMrpDox | 4 (2.35%) |
| RP-18 | AmxCtxCtrCfmCazCipLevCotNitAkGenCfsPitMrplpmDox | 3 (1.76%) |

Abbreviations: Amx, amoxycillin; Ctx, cefotaxime; Ctr, ceftriaxone; Cfm, cefixime; Caz, ceftazidime; Cot, cotrimoxazole; Nit, nitrofurantoin; Lev, levofloxacin; Ak, amikacin; Gen, gentamicin; Cfs, cefoperazone/sulbactam; Pit, piperacillin/tazobactam; Mrp, meropenem; lpm, imipenem; Dox, doxycycline.

Table 3 Resistance to Different Classes of Antibiotics Among β -Lactamase Producer and Non-Producer MDR Isolates

| No. of Class of Antibiotic Resistant | Antibiotics Resistance Pattern | No. of Strains | No. of Antibiotic Category | ESBL (n=95) | AmpC (n=15) | MBL (n=11) | ESBL+ AmpC (n=33) | ESBL +MBL (n=5) | MBL +AmpC (n=2) | MBL +KPC (n=1) | Non-producer (n=8) |
|--------------------------------------|--|----------------|----------------------------|-------------|-------------|------------|-------------------|-----------------|-----------------|----------------|--------------------|
| 3 | AmxCtxCttCfmCazCot | 7 | 3 | 7 | – | – | – | – | – | – | – |
| 4 | AmxCtxCttCfmCazCipLevMrp | 22 | 4 | 7 | 1 | – | – | – | – | – | 2 |
| | AmxCtxCttCfmCazCipMrp | | 4 | 7 | – | – | 1 | – | – | – | – |
| | AmxCtxCttCfmCazCipCot | | 4 | 2 | – | – | 2 | – | – | – | – |
| 5 | AmxCtxCttCfmCazCipLevCotMrp | 25 | 5 | 14 | – | – | – | – | – | – | – |
| | AmxCtxCttCfmCazCipLevCotDox | | 5 | 9 | – | – | 2 | – | – | – | – |
| 6 | AmxCtxCttCfmCazCipLevCotMrpDox | 49 | 6 | 18 | – | – | 7 | – | – | – | 1 |
| | AmxCtxCttCfmCazCipLevCotCfsMrp | | 5 | 6 | – | – | 1 | – | – | – | 2 |
| | AmxCtxCttCfmCazCipCotMrpDox | | 6 | 5 | – | – | 1 | – | – | – | – |
| | AmxCtxCttCfmCazCipLevCotNitMrp | | 6 | 4 | – | – | 1 | – | – | – | – |
| | AmxCtxCttCfmCazCipLevCotGenMrp | | 6 | 3 | – | – | 1 | – | – | – | – |
| 7 | AmxCtxCttCfmCazCipLevCfsPitMrpDox | 6 | 6 | 2 | 3 | – | 1 | – | – | – | – |
| 8 | AmxCtxCttCfmCazCipLevCotCfsPitMrpDox | 33 | 7 | 4 | 5 | – | 9 | 3 | 2 | – | 3 |
| | AmxCtxCttCfmCazCipLevCotGenCfsMrpDox | | 7 | 5 | 1 | – | 1 | – | – | – | – |
| 9 | AmxCtxCttCfmCazCipLevCotAkGenCfsPitMrpDox | 28 | 8 | – | 1 | 1 | 2 | – | – | – | – |
| | AmxCtxCttCfmCazCipLevCotNitCfsPitMrpDox | | 8 | 3 | 4 | 2 | 4 | 1 | – | – | – |
| | AmxCtxCttCfmCazCipLevCotAkGenCfsPitMrpDox | | 8 | – | – | 7 | – | – | – | – | – |
| 10 | AmxCtxCttCfmCazCipLevCotNitAkGenCfsPitMrpDox | 3 | 9 | – | – | 1 | – | 1 | – | 1 | – |

Abbreviations: Amx, amoxicillin; Ctx, cefotaxime; Ctr, ceftriaxone; Cfm, ceftazidime; Cot, cotrimoxazole; Nit, nitrofurantoin; Lev, levofloxacin; Ak, amikacin; Gen, gentamicin; Cfs, cefoperazone/sulbactam; Pit, piperacillin/tazobactam; Mrp, meropenem; lpm, imipenem; Dox, doxycycline.

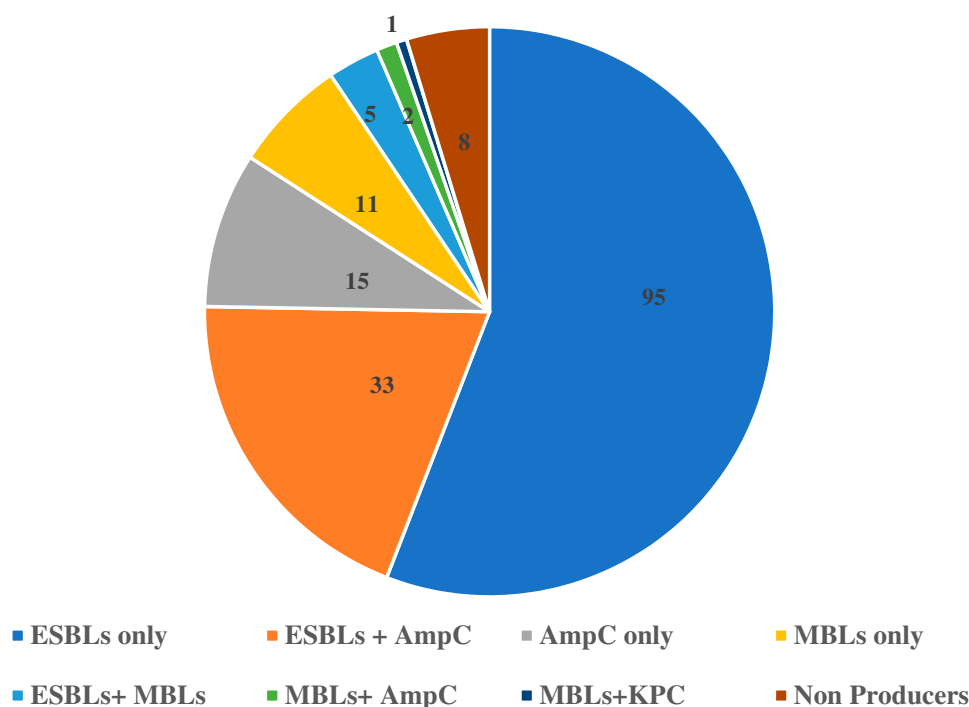
Beta-lactamase production among MDR *E. coli* isolates (n=170)

Figure 1 Distribution of different β -lactamases among MDR *E. coli* isolates.

that explored all patients or all the infectious diseases. In addition, the commonly used antibiotics in Nepal both at the hospital and over the counter include mostly oral penicillins and cephalosporins, which might explain the high resistance among these groups of antimicrobials.^{47,48}

More than 70% of the isolates were ESBLs producers, nearly half of the isolates were AmpCs producers and fewer percent of MBLs and KPCs producers were found in this study. The continuous exposure of bacteria to a variety of β -lactams has been well established for the production of β -lactamases, the most common mechanism of conferring β -lactams resistance among *Enterobacteriaceae*.⁸ The β -lactamase enzyme production mediated by both chromosomal and plasmid genes (subject to inducible expression) is a pivotal means of antibiotic resistance. These characteristic features are usually coded on transferable genes.⁸ β -Lactamase genes are usually found on plasmids encoding resistance to aminoglycosides, fluoroquinolones, sulfonamides, tetracyclines and other antibiotics,⁴⁹ thus leading to cross resistance. Pathogen producing ESBLs, AmpCs and carbapenemases like MBLs and KPCs leaves us with limited treatment options. Further, MBLs just like ESBLs and AmpCs producers can be transferred between species by plasmids.⁵⁰

In Nepal, there were limited studies demonstrating a high level of ESBLs producers among which *Enterobacteriaceae* were 28% to 67%.⁵¹ Most of the studies conducted showed that the ESBLs producers range as low as 22.3% to as high as 86.9%. Studies conducted in hospital settings reported 55% of ESBLs producers in Kathmandu,⁵¹ 27.7% in Pokhara²³ and 63.27% in Kathmandu Model Hospital.⁵² Previous studies from India have reported ESBLs productions in uropathogenic *E. coli* ranging from 18.5% to 60.7%.⁵³

AmpC β -lactamases are plasmid-mediated that hydrolyze all cephalosporins except cefepime and the carbapenems. In AmpCs, the inducible chromosomal genes are mobilized as plasmids.⁵⁴ In this study, the rate of AmpCs detection was 46.3%. The production of AmpCs in our hospital setting is higher compared to other studies that showed 12.6% at the tertiary care transplant center, in Kathmandu, Nepal,⁵⁵ 19.8% over a period of 6 months at various tertiary care hospitals in India,⁵⁶ 11.9% at a teaching hospital in Nigeria,⁵⁷ and 5% in three major hospitals of Iran.⁵⁸ The higher rate of AmpCs in this study might be due to the use of penicillin, cephalosporins and oxymino- β -lactams drugs as the primary choice of empiric therapy for the infections caused by Gram-negative bacteria.

Table 4 Plasmid Profile of MDR *E. coli* Isolates

| No. of Plasmids | Type | Molecular Weight (kb) | Plasmid Profile | No. of Isolates Showing Profile (%) | Total Number (%) |
|-----------------|--|--|---|--|------------------|
| 1 | 1a | >33.5 | 1 | 20 (11.76) | 20 (11.76) |
| 2 | 2a 2b 2c 2d 2e 2f 2g | >33.5, 33.5 >33.5 (2) >33.5, 15.0 >33.5, 9.4 >33.5, 10.1 >33.5, 6.0 >33.5, 4.8 | 2 3 4 5 6 7 8 | 19 (11.17) 12 (7.1) 10 (5.88) 9 (5.29) 8 (4.71) 5 (2.94) 4 (2.35) | 67 (39.41) |
| 3 | 3a 3b 3c 3d 3e 3f 3g 3h 3i 3j | >33.5, 15.0, 2.0 >33.5 (2), 3.0 >33.5, 6.7, 2.4 >33.5, 14.0, 1.5 >33.5 (2), 33.5 >33.5, 14.0, 9.0 >33.5 (2), 2.2 >33.5, 33.5, 2.1 >33.5, 9.0, 4.0 >33.5, 2.0, 1.5 | 9 10 11 12 13 14 15 16 17 18 | 6 (3.53) 5 (2.94) 4 (2.35) 3 (1.76) 2 (1.17) 2 (1.17) 2 (1.17) 2 (1.17) 2 (1.17) 1 (0.58) | 29 (17.05) |
| 4 | 4a 4b 4c 4d 4e 4f 4g 4h 4i | >33.5, 15.0, 4.0, 2.0 >33.5, 33.5, 4.8, 4.0 >33.5, 15.0, 4.0, 2.5 >33.5, 15.0, 7.0, 5.0 >33.5, 33.5, 6.3, 3.1 >33.5 (2), 2.6, 2.0 >33.5, 33.5, 14.8, 11.8 >33.5, 33.5, 2.0, 1.0 >33.5 (2), 33.5, 4.4 | 19 20 21 22 23 24 25 26 27 | 9 (5.29) 6 (3.53) 3 (1.76) 3 (1.76) 3 (1.76) 1 (0.58) 1 (0.58) 2 (1.17) 1 (0.58) | 29 (17.05) |
| 5 | 5a 5b 5c 5d 5e 5f 5g | >33.5 (2), 5.0, 3.1, 2.4 >33.5 (3), 33.5, 1.2 >33.5, 33.5, 2.0, 1.5, 1.0 >33.5, 33.5, 3.1, 2.0, 1.6 >33.5 (2), 33.5, 3.0, 2.0 33.5, 10.0, 7.4, 4.0, 3.0 >33.5, 15.0, 5.0, 4.4, 0.8 | 28 29 30 31 32 33 34 | 3 (1.76) 1 (0.58) 1 (0.58) 1 (0.58) 1 (0.58) 1 (0.58) 1 (0.58) | 9 (5.29) |
| 6 | 6a 6b 6c 6d 6e 6f 6g 6h 6i | >33.5, 10.6, 4.0, 3.0, 1.5, 1.2 >33.5 (2), 6.0, 3.0, 2.0, 1.5 >33.5 (2), 12.7, 9.0, 5.0, 2.5 >33.5 (2), 5.0, 4.8, 2.4, 2.1 >33.5 (3), 33.5, 3.0, 1.0 >33.5, 12.0, 5.5, 2.5, 1.9, 1.2 >33.5, 33.5, 4.0, 3.0, 2.0, 1.6 >33.5, 33.5, 7.0, 5.0, 4.0, 2.0 >33.5, 33.5, 12.7, 15.0, 3.0, 1.5 | 35 36 37 38 39 40 41 42 43 | 4 (2.35) 3 (1.76) 1 (0.58) 1 (0.58) 1 (0.58) 1 (0.58) 1 (0.58) 2 (1.17) 1 (0.58) | 15 (8.76) |
| 7 | 7a | >33.5, 33.5, 9.4, 3.0, 2.2, 2.0, 1.0 | 44 | 1 (0.58) | 1 (0.58) |

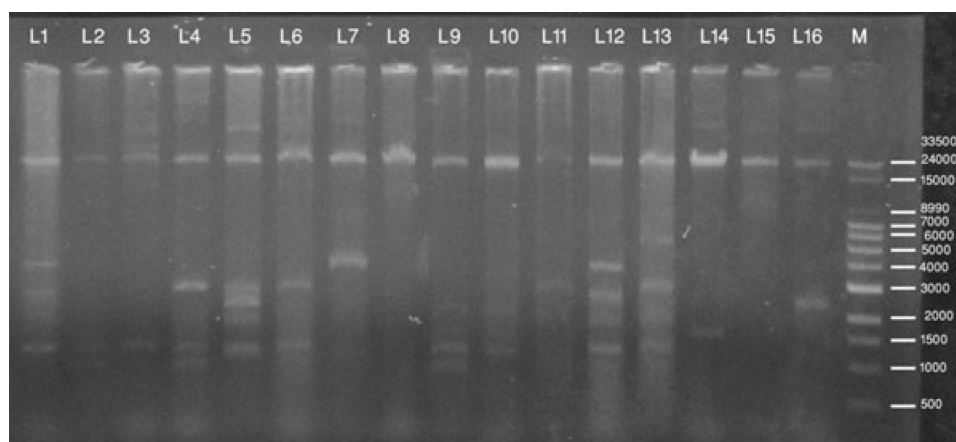
Table 5 Plasmid Profiling in Relation to β -Lactamase Production in MDR *E. coli* Isolates

| Plasmid Number | Molecular Weight | No. of Isolates | ESBL | AmpC | MBL | ESBL/AmpC | ESBL/MBL | MBL/AmpC | MBL/KPC | Non-producer |
|----------------|---|-----------------|------|------|-----|-----------|----------|----------|---------|--------------|
| 1 | >33.5 | 20 | 13 | 2 | – | 3 | – | – | – | 2 |
| 2 | >33.5, 33.5, 15.0, 10.1, 9.4, 6.0, 4.8 | 67 | 32 | 6 | 7 | 14 | – | 2 | 1 | 5 |
| 3 | >33.5, 33.5, 15.0, 14.0, 9.0, 6.7, 4.0, 3.0, 2.4, 2.2, 2.1, 2.0, 1.5 | 29 | 16 | 4 | 2 | 6 | 1 | – | – | – |
| 4 | >33.5, 33.5, 15.0, 14.8, 11.8, 7.0, 6.3, 5.0, 4.8, 4.4, 4.0, 3.1, 2.5, 2.6, 2.0, 1.0 | 29 | 21 | – | 1 | 5 | 2 | – | – | – |
| 5 | >33.5, 33.5, 15.0, 10.0, 7.4, 5.0, 4.4, 4.0, 3.8, 3.1, 3.0, 2.4, 1.6, 1.5, 1.2, 1.0, 0.8 | 9 | 5 | 1 | 1 | 1 | – | – | – | 1 |
| 6 | >33.5, 33.5, 12.7, 10.6, 9.0, 7.0, 6.0, 5.0, 4.8, 4.0, 3.0, 2.5, 2.4, 2.1, 2.0, 1.9, 1.6, 1.5, 1.2, 1.0 | 15 | 7 | 2 | – | 4 | 2 | – | – | – |
| 7 | >33.5, 33.5, 9.4, 3.0, 2.2, 2.0, 1.0 | 1 | 1 | – | – | – | – | – | – | – |
| Total isolates | | 170 | 95 | 15 | 11 | 33 | 5 | 2 | 1 | 8 |

Carbapenems are the ultimate resort for the treatment of isolates resistant to penicillin and cephalosporins.⁵⁹ Carbapenem resistance due to carbapenemase production was firstly discovered in 1988 in New York, America.⁶⁰ In our study, the rate of MBLs detection was 11.2%. Government medical college in Uttarakhand, India, reported 15.3% MBLs-producing isolates⁶¹ and a medical college in South India reported 13.4% MBLs-producing *E. coli*,⁶² which are almost consistent with this study. MBLs-producing *E. coli* were similar across other studies from India (2.87%), Dubai (0.3%) and Nigeria (41.2%).^{63–65} In this study, KPCs production was 0.6% in contrast to a study conducted in Iran which reported the prevalence of KPCs to be 1.4%.⁶⁴ This

particular difference could have been due to the variation in the settings.

A total of 41 (24.12%) isolates co-produced β -lactamase enzymes in this study, whereas other studies from our similar settings, with different clinical samples, have reported to be 6.2%.⁶⁵ However, a study from Punjab, India, showed a similar prevalence of 19.04%.⁶⁶ The co-existence of different classes of β -lactamases in a single bacterial isolate imposes diagnostic and treatment challenges with limited available drugs that are potentially toxic that include polymyxin and colistin.⁶² Furthermore, the outcomes for infected patients treated with such drugs remain unknown.⁶⁷

**Figure 2** Separation of plasmid DNA molecular weight on agarose gel stained with ethidium bromide (lanes 1–16: plasmid DNA of clinical isolates of MDR *E. coli*, lane (M) marker DNA – 33.5 kb DNA ladder).

E. coli isolates on plasmid profiling showed one plasmid >33.5 kb and was present in all the isolates. Many of the isolates possessed common plasmids of similar molecular size and these isolates also show the same β -lactamase production. Existence of common plasmid among the isolates implies the spread of resistant plasmid in the community. Plasmids of molecular size ranging from 0.8 kb to >33.5 kb were isolated from all the MDR isolates. Contrastingly, plasmid sizes ranging from 0.12 kb up to 65 kb were reported from West Nigeria (from 0.12 kb to 23 kb), Kathmandu (from 2.05 kb to >33.5 kb) and south-western Nigeria (from 11.8 kb to 33.5 kb).^{65,68,69}

Different plasmids can co-exist in the same host cell.³⁵ Based on the antibiotic resistance patterns, the high molecular weight plasmid seemed to consist most of the resistance genes as previous studies have also demonstrated that multi-antibiotic resistance is associated with higher molecular weight plasmids.^{70,71} Comparing the antibiotic pattern with the plasmid profile pattern does not offer much in linking specific antibiotic patterns with specific plasmid profiles. This suggests that the genes resistant to various antibiotic patterns were probably distributed in plasmids of various sizes as well as in the chromosomes.

Strengths and Limitations

This is the first study to explore the plasmid profile in correlation with different β -lactamases of uropathogenic *E. coli* in patients attending a tertiary hospital in Kathmandu. Constrained by the objective of the study, we did not collect information on socio-demographics of the patients, which could have provided vital information about the source and characteristics of the samples. This study was conducted in one tertiary hospital in Kathmandu; thus, the findings from this study may not be generalizable, although the findings are consistent with previous studies. This study relied on the plasmid profiles of the uropathogens to derive the results, adding genotypic information could have helped to determine the role and distribution of antibiotic resistance. Different buffer solutions used in agarose gel electrophoresis were prepared manually. Therefore, we found a poor resolution of the separated band of plasmid DNA, which is one of the major limitations of this study. Because of logistical region, we used alkaline lysis method for isolation of bacterial plasmid. As a conventional method, it can easily remove chromosomal DNA; however, chances of plasmid sharing could be one of the reasons for small size plasmid in our study. Future studies can provide more information by exploring the

plasmid profile together with the genotypic information. In future, studies conducted in multiple sites can be useful in providing more generalizable results.

Conclusions

This study has shown a high prevalence of beta-lactamases-producing *E. coli* in a tertiary hospital of Kathmandu. This clearly shows the urgency of the emerging resistance. To counteract such an emerging resistance, at first, detection of ESBLs producers should be routinely recommended in all tertiary hospitals including the peripheral laboratories of Nepal. Second, an antibiotic therapy guideline should be developed to scrutinize the use of antibiotics in hospitals including on-the-counter sale of antibiotics for infections such as UTI. In addition, profiling of the plasmid and chromosome of *E. coli* can be useful to ascertain the evolution and spread of antibiotic resistance among isolates.

Abbreviations

AmpCs BL, AmpC type β -lactamases; AST, antibiotic susceptibility test; ATCC, American Type Culture Collection; CLED, cystine–lactose–electrolyte-deficient agar; CLSI, Clinical and Laboratory Standard Institute; EDTA, ethylene diamine-tetraacetic acid; ESBL, extended-spectrum β -lactamase; IPD, inpatients department; KMH, Kathmandu Model Hospital; KPCs, *Klebsiella pneumoniae* carbapenemases; MBLss, metallo- β -lactamase; MDR, multidrug-resistant; MHA, Mueller Hinton Agar; MSU, mid-stream urine; NDM, New Delhi metallo β -lactamase; OPD, outpatients department; PBP, phenyl boronic acid; UTI, urinary tract infection; WHO, World Health Organization.

Data Sharing Statement

All data pertaining to this study are within the manuscript.

Ethical Approval and Consent

This study was ethically approved from institutional review committee (IRC); pfect-NEPAL. A written informed consent was obtained from each patient before their participation and collection of samples. In the case of illiterate participants, information was provided by reading the consent form in the presence of witnesses.

Acknowledgments

The authors would like thank Kantipur College of Medical Science and Kathmandu Model Hospital for providing an opportunity to conduct this study. The authors are also

grateful to all the patients and technical staff for their help during the study. We would like to express our gratitude to Mr. Gordon Tambellini, USA, for proof reading and edits.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Funding

This research was supported by Departmental fund of Kantipur College of Medical Sciences, Sitapaila, Kathmandu.

Disclosure

The authors declare that they have no competing interests.

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