Detection of NDM Variants ($bla_{NDM-1}$, $bla_{NDM-2}$, $bla_{NDM-3}$) from Carbapenem-Resistant Escherichia coli and Klebsiella pneumoniae: First Report from Nepal

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Background: Increasing burden of carbapenem resistance among Enterobacterales is attributable to their ability to produce carbapenemase enzymes like metallo-beta-lactamase (MBL), Klebsiella pneumoniae carbapenemase (KPC), and OXA-type. This study aimed to determine the prevalence of carbapenemases and MBL genes ($bla_{NDM-1}$, $bla_{NDM-1}$ and $bla_{NDM-3}$) among E. coli and K. pneumoniae isolates.

Methods: A total of 2474 urine samples collected during the study period (July–December 2017) were processed at the microbiology laboratory of Kathmandu Model Hospital, Kathmandu. Isolates of E. coli and K. pneumoniae were processed for antimicrobial susceptibility testing (AST) by disc diffusion method. Carbapenem-resistant isolates were subjected to Modified Hodge Test (MHT) for phenotypic confirmation, and inhibitor-based combined disc tests for the differentiation of carbapenemase (MBL and KPC). MBL-producing isolates were screened for NDM genes by polymerase chain reaction (PCR).

Results: Of the total urine samples processed, 19.5% (483/2474) showed the bacterial growth. E. coli (72.6%; 351/483) was the predominant isolate followed by K. pneumoniae (12.6%; 61/483). In AST, 4.4% (18/412) isolates of E. coli (15/351) and K. pneumonia (3/61) showed resistance towards carbapenems, while 1.7% (7/412) of the isolates was confirmed as carbapenem-resistant in MHT. In this study, all (3/3) the isolates of K. pneumoniae were KPC-producers, whereas 66.7% (10/15), 20% (3/15) and 13.3% (2/15) of the E. coli isolates were MBL, KPC and MBL/KPC (both)-producers, respectively. In PCR assay, 80% (8/10), 90% (9/10) and 100% (10/10) of the isolates were positive for $bla_{NDM-1}$, $bla_{NDM-1}$ and $bla_{NDM-3}$, respectively.

Conclusion: Presence of NDM genes among carbapenemase-producing isolates is indicative of potential spread of drug-resistant variants. This study recommends the implementation of molecular diagnostic facilities in clinical settings for proper infection control, which can optimize the treatment therapies, and curb the emergence and spread of drug-resistant pathogens.

Keywords: E. coli, K. pneumoniae, MBL, KPC, NDM variants

Background
Gram-negative bacteria, especially those falling under the family Enterobacterales (eg, Escherichia, Klebsiella, and Enterobacter) as well as pseudomonads and Acinetobacter serve as the potential pathogens in nosocomial and community-acquired infections in urinary and respiratory tracts, blood stream, intra-abdominal and surgical site infections. Some of these Gram-negative bacilli are also included in ESFAKE—a group of six nosocomial pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.) that are notorious for drug resistance and virulence. Among those species, Escherichia coli and Klebsiella spp., are the most frequently reported pathogens in developing countries with poor health care system.
Antibiotics are the empirical choice of treatment to counter such pathogenic strains of bacteria. However, despite the advent of effective antibiotic therapy, bacteria are constantly evolving noble mechanisms to evade the treatment regimen in question, giving rise to the global problem of antimicrobial resistance (AMR)—a condition in which pathogenic bacteria develop resistance to specific antibiotics to which they were at first sensitive. Infection management becomes even more onerous once a pathogenic strain develops multidrug resistance (MDR)—resistance towards at least one antimicrobial drug in three or more antimicrobial categories. These MDR strains are often referred as 'superbugs' or “nightmare bacteria” because of the reduced treatment options that are associated with a greater degree of morbidity and mortality and raised healthcare costs.

Globally, β-lactam—a group of antibiotics—are the most extensively used drugs against drug-resistant strains, which alone constitute about two-thirds of the total antibiotic prescriptions. This group of antibiotics comprises four major chemical classes: penicillins, cephalosporins, carbapenems, and monobactams, in which carbapenems (often deemed as the last resort drug) are the most effective against Enterobacteriaceae—the most prevalent group of bacteria encountered worldwide. However, several studies suggest the emergence and global spread of carbapenem-resistant Enterobacteriaceae (CRE), wreaking havoc in infection management, as the clinicians are left with little antibiotic options to counter MDR strains despite the latest advent of new classes of drugs such as beta-lactam/beta-lactam inhibitor (BL/BLIC) and cefiderocol.

Drug-resistant bacteria evade the β-lactam antibiotics by producing beta-lactamases—a group of hydrolytic enzymes that inactivates the drug before reaching penicillin-binding proteins (PBPs) located at the cytoplasmic membrane. This problem is further aggravated by the permeability defects coupled with the overexpression of AmpC or ESBL beta-lactamases. Extended-spectrum β-lactamases (ESBLs), AmpC, cephalosporinase, and carbapenemase are some of the representative and predominant families in the classification of β-lactamases. While all classes have been reported globally, their geographical distribution and prevalence may vary within and between the countries.

Carbapenemase are member of Ambler class A, class B and class D β-lactamases. Class A enzymes are serine carbapenemase (KPC, SME, IMI, NMS, and GES, inhibited by clavulanic acid), Class B are metallo-β-lactamases (MBLs) (IMP, VIM, NDM, SPM, GIM, and SIM, inhibited by metal chelators carbapenem) and Class D are oxacillinase-type (OXA types) carbapenemase. During hydrolysis of β-lactam ring (of β-lactam drugs), Class A and Class D carbapenemase require serine at their active sites, while MBL carbapenemase requires metal ion (often zinc) in their active sites. The emergence of class B carbapenemase (MBL) has posed the greatest challenge in treatment because they confer resistance to a wide range of drugs (carbapenems, cephalosporins, and penicillins) except for monobactams. Moreover, MBL is known for the acquisition of some rapidly evolving and spreading genes such as IMP, VIM, and NDM. The newest variant New Delhi metallo-β-lactamase (NDM) was first reported in 2009 in an isolate of K. pneumoniae from a Swedish traveler, admitted in hospitals in Delhi. A previous surveillance report collected from 40 countries has shown that NDM-type variants alone account for 44.2% of all MBL-producing Enterobacteriaceae. Aside from widespread presence in the Indian subcontinent, this variant is now endemic in Balkan countries, Northern Africa, and the Arabian Peninsula. Today, NDM variants are classified as NDM-1 through NDM-25, with NDM-1 and NDM-5 being commonly detected in Enterobacterales. Similarly, an important member of Ambler Class A beta-lactamase, namely Klebsiella pneumoniae carbapenemase (KPC) also remains prevalent in clinical specimens. Although, K. pneumoniae remains the principal reservoir for KPC, the enzyme has been identified in several Gram-negative bacilli. First reported in 1996 in the United States from an isolate of K. pneumoniae, KPC became cosmopolitan in the next few decades.

Early diagnosis and treatment are the pillars of infection management. In other words, precise detection and identification of resistant strains remains as the foundation step in combating AMR. However, diagnostic laboratories in the developing countries like Nepal suffer from the limitations in their capacity such as molecular characterization and detection of the pathogens thereby suggesting a need of more advanced facilities to reinforce prompt diagnosis of any causative agents. Even the expert clinicians fail to identify those rapidly evolving (or mutating) chromosomal and/or plasmid-encoded genes responsible for the expression of resistant enzymes, suggesting the need of an unabated research and development of noble methodology and tools. Furthermore, some of the diagnostic procedures in use are resource-intensive, often requiring the reagents that are not easily accessible. For these reasons, resistant strains in clinical samples...
go undetected and the overall prevalence remains underreported. To the best of our knowledge, despite an increasing burden of carbapenemase (especially MBL genes), there is a paucity of research studies in Nepal. Therefore, this study was carried out to determine the overall prevalence of KPC and MBL carbapenemase-producing Enterobacteriaceae (*E. coli* and *Klebsiella pneumoniae*) and further isolation of three variants of MBL carbapenemase, namely bla\textsubscript{NDM-1}, bla\textsubscript{NDM-2} and bla\textsubscript{NDM-3} in clinical samples collected at a tertiary care center of Kathmandu, Nepal. To the best of our knowledge, this is the first study conducted in Nepal that aimed to detect bla\textsubscript{NDM-2} variant among clinical isolates.

**Methods**

**Study Design, Study Site and Sample Population**

The hospital-based cross-sectional study was conducted over a period of 6 months (July–December 2017) at Kathmandu Model Hospital, Kathmandu, Nepal. A total of 2474 non-duplicated, clean-catch mid-stream urine samples were collected from the patients with suspected urinary tract infection (UTI). The study included patients of all ages and genders from outpatient departments (OPD) or admitted to the hospital. All of the study subjects were asked to provide written informed consent for their voluntary participation in the study. A well-structured questionnaire was administered to each patient to document their demographic information, clinical history and prior history of any medication. Patients with incomplete demographic information and those with ongoing antibiotic therapy were excluded from this study.

**Sample Collection and Transport**

Samples were aseptically collected adhering to the standard protocol. A sterile, dry, wide-mouthed and leak-proof container was administered to each patient to collect 10–20 mL of mid-stream urine sample. Once the sample was received, it was labeled and immediately delivered to the microbiology laboratory of the hospital for further processing. In case of unwanted delay, clinical samples were refrigerated at 4–6 °C.

**Bacteriological Culture of Clinical Specimens, Isolation and Identification of Isolates**

Urine samples were cultured semi-quantitatively on Cysteine lactose electrolyte deficient (CLED) agar (Hi Media, India) by using a standard calibrated loop. After inoculation, the culture plates were incubated with sufficient aeration at 37 °C overnight. Following incubation, culture plates were examined for the morphology of bacterial colonies (growth of microorganisms). Such colonies were counted and approximated by following standard guidelines. Plates showing more than or equal to $10^5$ colony-forming units (CFU)/mL of urine sample were considered as positive result for bacterial growth. Only the confirmed isolates of *E. coli* and *K. pneumoniae* were subjected for further processing.

**Antimicrobial Susceptibility Testing (AST)**

Bacterial isolates under study were processed for antibiotic susceptibility testing by using modified Kirby–Bauer disk diffusion method on Mueller–Hinton agar (MHA) (Hi-Media, India) (CLSI, 2016). Isolates were tested against the following antibiotic discs: amoxicillin (10µg), ceftriaxone (30µg), cefotaxime (30µg), ceftazidime (30µg), norfloxacin (10µg), ofloxacin (5µg), ciprofloxacin (5µg), nitrofurantoin (300µg), gentamicin (30µg), cotrimoxazole (25µg), amikacin (30µg), chloramphenicol (30µg), piperacillin/tazobactam (100/10µg), imipenem (10µg), meropenem (10µg), and cefoperazone sulbactam (75/30µg). The test results were interpreted on the basis of Clinical and Laboratory Standard Institute (CLSI) guidelines. Isolates showing resistance to at least one antibiotic of three or more classes of antibiotics were labeled as MDR.

**Screening for Carbapenemase Production**

In AST, bacterial isolates showing resistance to imipenem and meropenem with inhibition zone of 6–15 mm (or existence of colonies in a 16–18mm) were regarded as suspected carbapenemase-producers. Such isolates were further subjected for confirmatory test of carbapenemase production.
Phenotypic Confirmation of Carbapenemase Production

The production of carbapenemase was confirmed by using the modified Hodge test (MHT), as explained by CLSI guidelines of 2016. In this assay, 0.5 McFarland carbapenem-susceptible strain of *E. coli* ATCC 25922 was used as an indicator strain, which was prepared in broth (or saline) and diluted to the final dilution of 1:10 in broth (or saline). A lawn of the diluted strain was made on the agar plate and allowed to dry for 3–5 min. An antibiotic disk (10µg meropenem) was placed at the center of the MHA plate. Then, in a straight-line manner, the test organism was streaked from the edge of the disc to the periphery of the plate. The plate was then incubated aerobically at 37 °C for 16–24h, and examined. A clover leaf-like indentation of the indicator strain along the growth streak of the test organism within the disc diffusion zone was deemed as a positive (confirmatory) test result, while the absence of such indentation of the indicator strain along the growth of the test organism was reported as negative.

Phenotypic Differentiation of MBL, KPC and MBL/KPC Production

Inhibitor-based combined-disc tests were used in the differentiation of MBL and/or KPC production. In this assay, carbapenem (10µg meropenem) disc alone and with 20µL (400µg) of phenylboronic acid (PBA) were used to detect KPC production. An increase in the diameter of the zone of inhibition (ZOI) by ≥5mm in meropenem disc supplemented with PBA (meropenem+ PBA) than the one without combination (meropenem only) was confirmed as the positive test result. Similarly, two meropenem discs, one without combination and another disc containing 10µL of 0.1 M ethylene diamine tetra acetate acid (EDTA) were used to confirm MBL production. An increase in ZOI of > 5mm in combined discs than that in single disc was reported as confirmed MBL-producers. Likewise, a ZOI of meropenem disc alone was used to compare the ZOI of another disc supplemented with PBA and EDTA (meropenem+EDTA+PBA). If the ZOI of the latter disc was increased by <5mm, the test is confirmed positive for the production of MBL and KPC simultaneously.

Thus, confirmed carbapenemase-producing isolates were preserved at −20°C in the tryptic soy broth (TSB) with 20% glycerol (TSB+glycerol) until further processing for molecular assay.

Extraction of Plasmid DNA

One to two isolated colonies of *E. coli* and *K. pneumoniae* were separately inoculated in 3mL of Luria–Bertani (LB) broth and incubated aerobically at 37°C for 18–24 hours. The pure culture was further treated under alkaline-lysis method to obtain plasmid DNA. The extracted plasmids were then suspended in the Tris-EDTA (TE) buffer and stored at −20°C until further analysis.

Amplification of Gene (*bla*<sub>NDM</sub>)

The carbapenemase genes (*bla*<sub>NDM-1</sub>, *bla*<sub>NDM-2</sub>, *bla*<sub>NDM-3</sub>) under question were amplified by the PCR assay. The primers used with their sizes are illustrated in Table 1. The reaction mixture for the PCR (5. HOT FIREPol Blend Master Mix Ready to Load, Solis BioDyne, Estonia) was prepared as follows: 13µL of master mixture, 5µL of distilled water, 0.5µL each of the forward and reverse primers and 6µL of the extracted plasmid was added in individual amplification tubes to make up the total volume to 25µL. Required PCR cycles, temperature and times were followed on the basis of manufacturer’s guidelines. Obtained PCR products were further processed for visualization.

Detection of PCR Products by Gel Electrophoresis

The PCR products were visualized by using gel-electrophoresis in 1.5% (W/V) tris-acetate-EDTA (TAE) agarose gel stained with 0.1 µL dye (ethidium bromide). Once the gel was ready for use, 1µL of 100bp DNA ladder (SoCibiodyne), 3 µL of negative control, positive control and PCR amplicons were added to the first, second, third and remaining wells respectively. The prepared gel-system was processed for photo documentation and the results were analyzed. The well-characterized *E. coli* isolates carrying *bla*<sub>NDM-1</sub> gene was taken as the positive control in this assay, while negative control was devoid of any DNA.

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Quality Control
All the laboratory assays were conducted under strict adherence to the aseptic conditions. Laboratory equipment, culture media and reagents were regularly monitored for the parameters like temperature, storage conditions, and expiry date wherever applicable. Purity plates were used to ensure aseptic conditions during biochemical tests. Control strains of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used to ensure quality control during AST.

Data Management and Analysis
Collected data were analyzed using the statistical package for social science (SPSS) software version 24.0. Associations of the demographic variables were explored using the chi-square ($\chi^2$) test at 5% confidence interval. The p-value <0.05 was assumed to be significant for the association of variables.

Results
Growth Rate of Microorganisms Among Clinical Samples
Among total urine samples processed for culture, 19.5% (483/2474) were positive for bacterial growth, while 80.5% (1991/2474) were negative. This result is illustrated in Figure 1.

| **Table 1** Specific Primers Used in the Study for the Amplification of the Target Gene |
| Name of the Primer | Target Gene | Amplicon (bp) | Sequence 5’–3’ | Reference |
|--------------------|-------------|---------------|----------------|-----------|
| NDM-1 F            | *bla*NDM-1  | 476           | GGGCAGTCGCTTCCAACGGT GTAGTGCTCAGTGTCGGCAT | [32]       |
| NDM-1 R            | *bla*NDM-2  | 984           | CTCCTGCACATCGAAATCGC CACCTCATGGTGAATTCCGC | [33]       |
| NDM-2 F            | *bla*NDM-3  | 274           | AATACCTTGAGGGGCGGCAAA CCTGGACCAATGACCAGACC | [34]       |
| NDM-2 R            |             |               |                 |           |
| NDM-3 F            |             |               |                 |           |
| NDM-3 R            |             |               |                 |           |

Abbreviations: A, adenine; T, thymine; C, cytosine; G, guanine.

Figure 1 Growth rate in urine specimens.
In total isolates, *E. coli* was the most predominant isolate with prevalence rate of 72.7% (351/483) followed by *K. pneumoniae* (12.6%; 61/483), *Enterococcus faecalis* (4.3%; 21/483), and CONS (3.3%; 16/483). The distribution of entire isolates is presented in Figure 2.

Out of 412 isolates of *E. coli* (85.2%; 351/412) and *K. pneumoniae* (14.8%; 61/412), 42.5% (n=175) isolates were isolated from male (153 *E. coli* and 22 *K. pneumoniae*) and 57.5% (n=237) were (198 *E. coli* and 39 *K. pneumoniae*) from female patients. There was no significant association of culture positivity rate with gender of patients (p=0.27).

Similarly, isolates were obtained more frequently from the patients of young age-group of 16–30 years having the rates of 31.1% (109/351) and 24.6% (15/61) for *E. coli* and *K. pneumoniae*, respectively. While the lowest number of isolates was seen among the older age-group above 75 years with the rates of 5.4% and 1.6% for *E. coli* and *K. pneumoniae*, respectively. The association between culture positivity of *E. coli* and *K. pneumoniae* isolates with the age group was not statistically significant (p=0.470). Likewise, 88.3% (310/351) of the *E. coli* isolates and 78.7% (48/61) of the *K. pneumoniae* isolates were obtained from outpatients, while the remaining fractions were from inpatients. There was a significant association of the type of patient enrolled (inpatients or OPD) with the rate of isolation of bacteria under study (p=0.04). The distribution of the isolates according to the gender, age and type of dwelling (OPD or IPD) is detailed in Table 2.

### Table 2 Distribution of *E. coli* and *Klebsiella pneumoniae* According to Gender, Age and Type of the Patients

| Character                   | *E. coli* | *Klebsiella pneumoniae* | Total | p-value |
|-----------------------------|-----------|-------------------------|-------|---------|
|                             | N  | %  | N  | %  | N  | %  |     |
| **Gender**                  |    |    |    |    |     |     |      |
| Male                        | 153| 43.6| 22 | 36.1| 175| 42.5| 0.27 |
| Female                      | 198| 56.4| 39 | 63.9| 237| 57.5|       |
| **Age group (in years)**    |    |    |    |    |     |     |      |
| 0–15                        | 40 | 11.4| 11 | 18  | 51 | 12.4| 0.47 |
| 16–30                       | 109| 31.1| 15 | 24.6| 124| 30.1|       |
| 31–45                       | 78 | 22.2| 13 | 21.3| 91 | 22.1|       |
| 46–60                       | 61 | 17.4| 13 | 21.3| 74 | 18  |       |
| 61–75                       | 44 | 12.5| 8  | 13.1| 52 | 12.5|       |
| >75                         | 19 | 5.4 | 1  | 1.6 | 20 | 4.9 |       |
| **Type of patients**        |    |    |    |    |     |     |      |
| Out patients                | 310| 88.3| 48 | 78.7| 358| 86.9| 0.04 |
| In patients                 | 41 | 11.7| 13 | 21.3| 54 | 13.1|       |
Out of 22 antibiotics tested, nitrofurantoin was the most effective drug against *E. coli* as 90.9% (319/351) of the isolates were susceptible towards this drug, whereas gentamicin was the most effective against 90.2% (55/61) of the *K. pneumoniae* isolates. Both of the isolates showed the least susceptibility towards amoxicillin (Table 3).

**Prevalence of Carbapenem-Resistant *E. coli* and *K. pneumoniae* Isolates**

Of the total isolates, 4.4% (18/412) were preliminarily screened as carbapenem resistant. Among the resistant bacteria, 15 (83.3%) and 3 (16.7%) were the isolates of *E. coli* and *K. pneumoniae* respectively. In the confirmatory assay by MHT, 38.9% isolates (7/18) were confirmed as carbapenemase-producers, of which four (57.1%) were *E. coli* and three (42.3%) were *K. pneumoniae* (Table 4).

### Table 3 Antibiotic Susceptibility Profile of the Isolates

| Antibiotics         | *E. coli* |   |   | *K. pneumoniae* |   |   |
|---------------------|-----------|---|---|-----------------|---|---|
|                     | Sensitive | Intermediate | Resistant | Sensitive | Intermediate | Resistant |
| Gentamicin (30µg)   | 295 (84.0) | 0 | 56 (16.0) | 55 (90.2) | 1 (1.6) | 5 (8.2) |
| Nitrofurantoin (300µg) | 319 (90.9) | 3 (0.9) | 29 (8.3) | 3 (4.9) | 5 (8.2) | 53 (86.9) |
| Levofloxacin        | 205 (58.4) | 4 (1.1) | 142 (40.5) | 53 (86.9) | 0 | 8 (13.1) |
| Ofloxacin (5 µg)    | 195 (55.6) | 4 (1.1) | 152 (43.3) | 52 (85.2) | 0 | 9 (14.8) |
| Norfloxacin (5 µg)  | 192 (54.7) | 1 (0.3) | 158 (45.0) | 50 (82.0) | 0 | 11 (18.0) |
| Cefixime            | 182 (51.9) | 2 (0.6) | 167 (47.6) | 29 (47.5) | 0 | 32 (52.5) |
| Cefotaxime          | 182 (52.0) | 4 (1.1) | 164 (45.9) | 31 (50.8) | 0 | 30 (49.2) |
| Cotrimoxazole (25 µg) | 179 (51.1) | 0 | 171 (48.9) | 43 (70.9) | 0 | 18 (29.5) |
| Amoxicillin (10 µg) | 124 (35.3) | 2 (0.6) | 225 (64.1) | 2 (3.3) | 0 | 59 (96.7) |
| Amikacin (µg)       | 92 (78.6) | 3 (2.6) | 22 (19.0) | 12 (75.0) | 0 | 4 (25.0) |
| Doxycycline         | 57 (48.7) | 0 | 60 (51.3) | 12 (75.0) | 0 | 4 (25.0) |
| Amoxiclave          | 11 (9.3) | 0 | 107 (90.7) | 0 | 1 (1.6) | 15 (93.8) |
| Cefazidime          | 11 (9.6) | 0 | 103 (90.4) | 4 (25.0) | 0 | 12 (75.0) |
| Cefepime            | 12 (15.6) | 3 (3.9) | 62 (80.5) | 6 (75.0) | 0 | 10 (25.0) |
| Chloramphenicol (30 µg) | 68 (58.6) | 5 (4.3) | 43 (37.1) | 8 (50.0) | 0 | 8 (50.0) |
| Cefoperazone sulbactam | 60 (51.7) | 0 | 56 (48.3) | 6 (75.0) | 0 | 10 (25.0) |
| Piperacillin/tazobactam (100/10 µg) | 87 (74.4) | 3 (2.6) | 27 (23.1) | 10 (62.5) | 1 (6.3) | 5 (31.3) |
| Imipenem (10 µg)    | 103 (88.0) | 0 | 14 (12.0) | 13 (81.3) | 0 | 3 (18.8) |
| Meropenem (10 µg)   | 102 (87.2) | 0 | 15 (12.8) | 13 (81.3) | 0 | 3 (18.8) |
| Tigecycline         | 15 (100) | 0 | 0 | 3 (100) | 0 | 0 |
| Polymyxin           | 15 (100) | 0 | 0 | 3 (100) | 0 | 0 |
| Colistin            | 15 (100) | 0 | 0 | 3 (100) | 0 | 0 |

**Antibiogram of *E. coli* and *K. pneumoniae***

Out of 22 antibiotics tested, nitrofurantoin was the most effective drug against *E. coli* as 90.9% (319/351) of the isolates were susceptible towards this drug, whereas gentamicin was the most effective against 90.2% (55/61) of the *K. pneumoniae* isolates. Both of the isolates showed the least susceptibility towards amoxicillin (Table 3).

**Table 4 Carbapenem Resistant and Carbapenemase Production in *E. coli* and *K. pneumoniae***

| Bacteria       | Screening Test Carbapenem Susceptibility | Confirmedatory Test Modified Hodges Test |
|----------------|----------------------------------------|----------------------------------------|
|                | Resistant | Susceptible | Positive | Negative |
|                | N (%)     | N (%)       | N (%)    | N (%)    |
| *E. coli*      | 15 (83.3) | 336 (85.3)  | 4 (57.1) | 11 (100) |
| *K. pneumoniae* | 3 (16.7)  | 58 (14.7)   | 3 (42.9) | 0 (100)  |
| Total          | 18        | 394         | 7        | 11       |
Distribution of MBL, KPC and MBL/KPC-Producing Isolates

Among 18 carbapenem-resistant isolates, 15 (83.3%) were E. coli and 3 (16.4%) were K. pneumoniae. Of the 15 carbapenemase-producing E. coli isolates, 10 (55.5%) were MBL-producers, 3 (20.0%) were KPC-producers and 2 (11.1%) were both KPC and MBL-producers. On the other hand, all of the three carbapenem-resistant isolates of K. pneumoniae were KPC-producers while none of them were MBL-producers (Figure 3).

Antibiogram of MBL-Producing E. coli Isolates

MBL-producing E. coli exhibited an increased resistance towards quinolones (100%), β-lactams (100%), cefeporazone sulbactam (100%) while MBL non-producing isolates showed greater degree of resistance towards amoxicillin (63.0%), followed by cotrimoxazole, cefixime, and cefotaxime. The detailed result is shown in Table 5.

The Prevalence of NDM Variants (1, 2 and 3) in E. coli Isolates

In the PCR assay, NDM genes were screened among MBL-producing isolates of E. coli. The amplified NDM variants with their amplicon sizes of 476 bp (blaNDM-1), 984 bp (blaNDM-2) and 274 bp (blaNDM-3) were detected (Figure 4). Of the total (10) MBL-producing isolates, respectively, 8, 9 and all of them tested positive for blaNDM-1, blaNDM-2, blaNDM-3 (Figure 5).

Discussion

Overall Findings

The problems of AMR even to the last resort treatment—carbapenems—are surging globally. Rampant misuse and overuse of antibiotics has been identified as the principal cause of the drug resistance among pathogens in low-middle-income countries (LMICs) where proper infection control and antibiotic stewardships are seriously lacking.\(^{37}\) This emergence and spread of resistant bacteria is further aggravated by limitations in the early detection and management in the diagnostic laboratories in such developing countries.\(^{38}\) Selective pressure from the inappropriate use of carbapenems has led to the emergence of carbapenem-resistant Enterobacteriaceae.\(^{7}\) The Enterobacteriaceae constitute the principal members of pathogens, present in virtually all prevalent infections caused by Gram-negative bacteria (GNB). More specifically, Enterobacterales like E. coli and K. pneumoniae are the chief agents isolated from infections mediated by GNB.\(^{39}\) Although recognized for irreplaceable choice in bacterial infections, antibiotics are losing their efficacy day by day, leading the clinical world to the fear of “no antibiotic era”.\(^{40}\) Next to irrational practices of prescription and use of antibiotics, acquisition and transfer of resistant genes (responsible for expression of resistant enzymes) from multiple
reservoirs of pathogenic bacteria, environment, and animals are recommended as the chief cause of an unabated spread of AMR worldwide. This study was conducted to identify carbapenem-resistant uropathogenic \textit{E. coli} and \textit{K. pneumoniae} and possible acquisition of MBL carbapenemase genes (NDM variants) among such strains so that this study could become a valuable reference to figure out overall prevalence of drug resistance in the study region. In our study, almost one-fifth of the cultured samples were positive for significant bacterial growth Comparable results were observed in the previous studies conducted in Nepal while some other studies reported higher growth rate. In some other studies, however, there was a marked observation of the low growth rate. The varied growth positive rates among samples in the aforementioned studies might have been influenced by a number of parameters such as prior antibiotic therapy in the study subjects, presence of slow-growing bacteria in samples, and severity of disease manifestations. In concordance with global trend, \textit{E. coli} and \textit{K. pneumoniae} were the main causes of UTI in this study. Similar findings on the predominance of these two bacteria from urine samples were reported from International Children Friendship Hospital; Universal College of Medical Sciences Bhairahawa; Kathmandu Model Hospital; Human Organ Transplant Center, Alka Hospital, Lalitpur, and Nobel Medical College, Biratnagar. Higher incidence of these bacteria as sole representative of Enterobacteriaceae of GNB in urine samples can be attributed to their strong affinity (attachment) to the uroepithelium. In addition, they are able to colonize in the urogenital mucosa with adhesins, pili, fimbriae and P-blood group phenotype receptor. In this study, UTI was prevalent among female patients as compared to males. Aligning to a larger sample volume of women, the prevalence of uropathogens was also higher among them. Similar results were documented in some previous studies. In these all studies, the prevalence is even concentrated among women of the adult age group (16–45) during which period they are more sexually active. Higher incidence UTI and uropathogens among the female population is attributed a number of factors such as complex physiological status of their bodies, shorter length of the urinary tract, and proximity of the anus to the urethral opening.

### Table 5 Resistance Pattern Among MBL-Producing and Non-Producing \textit{E. coli}

| S.No. | Antibiotic | Resistance Pattern of MBL and Non-MBL Producer |
|-------|------------|-----------------------------------------------|
|       |            | MBL Producer N=10 n (%) | MBL Non-Producer N=341 n (%) | p-value |
| 1     | GEN        | 5 (50.0) | 51 (15.0) | 0.012 |
| 2     | NIT        | 4 (40.0) | 25 (7.3)  | 0.001 |
| 3     | LE         | 10 (100) | 132 (38.7)| 0.0001 |
| 4     | OF         | 10 (100) | 142 (41.6)| 0.001 |
| 5     | NX         | 10 (100) | 148 (43.4)| 0.002 |
| 6     | AMX        | 10 (100) | 215 (63.0)| 0.060 |
| 7     | COT        | 9 (90.0) | 162 (48.0)| 0.009 |
| 8     | CFM        | 10 (100) | 157 (46.0)| 0.004 |
| 9     | CTX        | 9 (90.0) | 155 (45.6)| 0.024 |
| 10    | AK         | 7 (70.0) | 15 (4.4)  | 0.0001 |
| 11    | AMC        | 10 (100) | 97 (28.4)| 0.361 |
| 12    | CAZ        | 10 (100) | 93 (27.3)| 0.380 |
| 13    | CPM        | 5 (50.0) | 57 (17.0)| 0.524 |
| 14    | PTZ        | 10 (100) | 17 (5.0)  | 0.0001 |
| 15    | DOX        | 7 (70.0) | 53 (16.0)| 0.205 |
| 16    | IMP        | 9 (90.0) | 5 (2.0)  | 0.0001 |
| 17    | MRP        | 10 (100) | 5 (2.0)  | 0.0001 |
| 18    | CFS        | 10 (100) | 46 (13.9)| 0.0001 |
| 19    | CX         | 10 (100) | 33 (10.0)| 0.0001 |
Antimicrobial Susceptibility Assay and Prevalence of Carbapenem Drug Resistance

In this study, a majority of the E. coli and K. pneumoniae isolates exhibited resistance against commonly prescribed broad-spectrum antibiotics. On the other hand, nitrofurantoin was an effective drug followed by gentamicin against most
(90.9% and 84% respectively) of the isolates of *E. coli*. This finding concords with some of the previous reports.\textsuperscript{59–61} Higher efficacy of this drug against uropathogenic *E. coli* may be explained by their narrow spectrum of activity, activity even in low or undetected serum concentration, and limited contact with bacteria dwelling outside the urinary tract.\textsuperscript{62} Gentamicin is restrictively used in community care settings because of their injectable nature which explains its sensitivity even in these times of uncontrolled AMR.\textsuperscript{63} Similarly, *K. pneumoniae* isolates were more susceptible to aminoglycosides followed by fluoroquinolones in this study. This finding is similar to the one previous report.\textsuperscript{64} *K. pneumoniae* isolates were least susceptible to amoxicillin (3.3%) followed by nitrofurantoin (4.9%) whereas in a previous study they were more susceptible to nitrofurantoin (81.6%).\textsuperscript{65} Like *K. pneumoniae*, *E. coli* were also least susceptible to amoxicillin; this finding resembles with a prior study by Randrianirina et al.\textsuperscript{66} It may be due to the fact that amoxicillin is a first-line drug which is easily hydrolyzed by the bacterial enzymes and offers less in the treatment of the infections caused by GNB. Documenting the plight of resistance to carbapenem was the theme of this study in which 4.4% isolates of the *E. coli* and *K. pneumoniae* were resistant against this drug. This finding is in line with a study by Cai et al\textsuperscript{67} which reported a rate of 4.5% while a study conducted in Nepal reported a higher rate of 9.1%.\textsuperscript{32} However, Liang et al\textsuperscript{68} revealed a very low-level of carbapenem resistance in *E. coli* (5/1014 isolates). There was a marked distinction in the number of resistant isolates of *E. coli* and *K. pneumoniae*, the former exhibiting a higher number; however, the rate was similar having 4.3% and 4.9%, respectively.

**Prevalence of MBL and KPC Carbapenemase**

Enterobacteriaceae exhibit the resistance to carbapenems by three possible mechanisms: efflux pump overactivity, porin loss (mutation), and production of carbapenemase enzymes. The former mechanisms are basically associated with multidrug resistance, while carbapenemase are more specific to carbapenem resistance. Nevertheless, the production of this enzyme (or types of this enzyme) is considered as the main mechanism of resistance among CRE.\textsuperscript{69} Regardless of their types, carbapenem resistance should be taken seriously for the management of infections. However, defining (or classifying) the carbapenemase has been recognized to possess epidemiological values.\textsuperscript{30} Several tests such as MHT, both boronic acid-based and EDTA-based inhibition methods, Carba NP tests, and detection of OXA type (OXA-48) (depending upon the high resistance to temocillin) have been proposed for the phenotypic detection of Carbapenemase.\textsuperscript{70} In this study, the rate detection of carbapenemase by nonspecific MHT was 38.9%, which was comparable to a previous study by Ramana et al.\textsuperscript{71} There was a marked deviation in the positivity rate by MHT and by combined disc tests, the latter showing a higher rate of production of at least one type of carbapenemase under study. The rate of acquisition of carbapenemase genes was further higher in genotypic methods (PCR in this study), suggesting the higher sensitivity and specificity of molecular detection techniques as seen in some earlier studies.\textsuperscript{72} The MHT test used in this study cannot differentiate the various classes of carbapenemase and suffers from the limitation of having lower sensitivity and specificity. In other words, not all carbapenemase-producing isolates of CRE appear as positive in MHT are carbapenem producers, and those isolates detected as negative in the same test may be detected with carbapenem resistance mechanisms other than carbapenemase production.\textsuperscript{69}

Corroborating the necessity of classifying the carbapenemase for epidemiological purpose, one of the specific objectives of our study was to detect the types of carbapenemase (MBL, KPC and MBL/KPC) in carbapenem-resistant isolates. Among 15 *E. coli* isolates in this study, 10 (66.7%) were MBL producers; 3 (20%) were KPC producers and the remaining 2 (13.3%) were MBL/KPC producer, whereas all the 3e isolates of *K. pneumoniae* were KPC producers in this study. This result concurred with a previous study conducted by Birgy et al,\textsuperscript{73} in which 30 genotypically characterized carbapenem-resistant Enterobacteriaceae: 9 (30%) were MBL producer, 7 (23.3%) were KPC producer and 6 (20%) were MBL/KPC producer. Another study conducted in the Human Organ Transplant Center reported lower incidence of MBL and KPC with 29.5% and 11.4% respectively among uropathogenic strains.

Metallo-\(\beta\)–lactamases (MBLs) have been identified from clinical isolates worldwide with increasing frequency for over the past few years.\textsuperscript{8} This study indicated a high incidence of MBL-producing *E. coli* (55.5%) in urine samples. This result is in harmony with the finding reported in Pakistan which indicated a high (33.6%) incidence of MBL-producing *E. coli*.\textsuperscript{74} However, a study from India in the same year reported a lower (7.6%) incidence of MBL-producing *E. coli*.\textsuperscript{75} In general, production of MBL in Enterobacteriaceae isolates currently follows an increasing prevalence pattern and the
prevalence rate may vary greatly within and between geographical locations. The increasing prevalence of ESBL-producing pathogens in Nepal\textsuperscript{4,39,44,51,76,77} coupled with a higher frequency of prescription and use of carbapenem antibiotics may be the driving factors behind the increasing incidence of carbapenemase-producing organisms.

**Drug-Resistance Pattern of MBL-Producers and Acquisition of NDM-Variants**

In this study, again the AST of MBL-producing \textit{E. coli} was performed in which the antibiotic resistance of MBL-producing \textit{E. coli} was higher than that of MBL non-producing isolates. All the MBL-producing isolates were resistant towards fluoroquinolones, \(\beta\)-lactams and cefepime/azulactam, which is in accordance with the study conducted by Al-agamy et al reporting the rate of resistant as 100% among enzyme-producers.\textsuperscript{78}

All the phenotypically confirmed MBL-producing \textit{E. coli} isolates were subjected to polymerase chain reaction (PCR) for possible acquisition of NDM variants. In the PCR assay, 80% of the isolates tested positive for all the three variants, suggesting an alarming rate of acquisition of resistant variants. As this was the first study in Nepal to detect NDM-2 variant, there was an increasing rate of other NDM variants as compared to the previous studies.\textsuperscript{10,34,79} PCR-based molecular tests are remarkable for high sensitivity and specificity, so are recommended as reference tool for genotypic identification. Therefore, carbapenem-sensitive isolates in this study also could have possessed carbapenemase genes which could have been shielded in non-specific phenotypic tests. Hence, sensitive and reliable tests like PCR are recommended for all isolates in further studies.

**Clinical and Policy Implications/Future Directions**

The emergence and spread of MDR pathogens, especially CRE as the most predominant aetiologic agent in infectious diseases is culminating as a major public health threat, not only limited to Nepal but all over the globe. The infections caused by these notorious pathogens are getting very difficult to manage with available drugs, leading the world to the nightmare of the “no antibiotic era”. Irrational practice of prescription and use of antibiotics, over-stretched burden on healthcare systems, lack of sanitation and poor infection control, rising population density, and globalization are recognized as some major driving forces behind the unabated spread of AMR.\textsuperscript{37} The selective pressure on drugs like carbapenems and colistin has not spared any drug that is unaffected by resistance. Therefore, the findings of the studies (including this) warrant the immediate need of early detection and management of the pathogen so that appropriate and accurate treatment therapy can be ensured to check the further proliferation of such strains. In addition, findings of this study are suggestive of the need of the routine monitoring and surveillance of AMR across the country, and augmentation of laboratory capacity to facilitate the detection of resistant genes can assist physicians and policymakers to execute the apt measures required to address the problem. Furthermore, jettisoning the practice of over-the-counter (OTC) use of drugs by imposing proper restrictions and periodic implementation of antibiotic stewardship programs in healthcare settings can also be a better option to buttress the rational use of drugs throughout the country.

**Strengths and Limitations**

To the best of our information obtained in literature review, it is the first study which pursued to determine \(\text{bla}_{\text{NDM-2}}\) genes in clinical samples. Therefore, this study is one of a handful of studies conducted in Nepal, which aimed to determine the prevalence of carbapenemase encoding NDM variants in uropathogenic strains of \textit{E. coli} and \textit{K. pneumoniae}. This study may serve as a valuable reference for clinicians, research scientists and policymakers to figure out the factual plight of the AMR in the country. In addition, the finding of the study can be pivotal in antibiotic stewardship programs to promote rational use of drugs in infectious diseases. Aside from its broad scopes in scientific and medical arena, this study suffers from some of the notable limitations. While the study strived to provide an in-depth perspective in carbapenem resistance mediated by carbapenemase (especially MBL carbapenemase), the relatively small sample size and the lack of multiple genotypes (other than NDM) seriously overlooked the actual burden of drug resistance. In addition, we could not perform whole-genome sequencing due to limitations in the resources and funding. Although MHT is not the best approach in phenotypic detection of carbapenemase, we relied on the same due to resource inaccessibility to other noble and more reliable techniques like carbaNP, carbapenem inactivation and blu carba tests.
Furthermore, this study cannot tell the origin and transferability of resistant genotypes detected in the study. Therefore, future studies are recommended to cover multiple healthcare and community settings for larger sample sizes, and employ more sensitive and specific diagnostic tests to detect all possible Ambler class carbapenemase and genes encoding them.

**Conclusion**
This study showed the substantial presence of MBL and/or KPC-producing isolates along with acquisition of NDM variants among uropathogens. This is an alarming risk because these multidrug-resistant bacteria can disseminate rapidly, thereby putting an end to our current pharmacopoeia. Therefore, early identification of NDM-related infections and prevention of their spread by implementing screening, hygiene measures and isolation of the carriers are needed. In addition, installation of advanced diagnostic facilities and assurance of rational use of antibiotics (guided by AST) can mitigate the emergence and spread of AMR.

**Abbreviations**
AMR, antimicrobial resistance; AST, antibiotic susceptibility test; ATCC, American Type Culture Collection; bla, gene-encoding β-lactamase; CLSI, Clinical and Laboratory Standard Institute; CRE, carbapenemase-resistant Enterobacteriaceae; CRKP, carbapenem-resistant *Klebsiella pneumonia*; ESBL, extended spectrum beta-lactamases; IMP, imipenemase; KPC, *Klebsiella pneumoniae* carbapenemase; LB, lysogeny broth; MA, MacConkey agar; MBL, metallo-β-Lactamases; MDR, multidrug resistance; MHA, Mueller–Hinton agar; MHT, modified Hodge test; MR, methyl red; NDM, New Delhi metallo-beta-lactamase; OPD, outpatient department; OXA, oxacillinase-hydrolyzing β-lactamase; p, probability; PBP, penicillin-binding protein; PCR, polymerase chain reaction; SHV, sulfhydryl variable β-lactamase; SPSS, Statistical Package for the Social Science; TEM, Temoniera β-lactamase; VIM, Verona integron-encoded metallo-β-lactamase; WHO, World Health Organization.

**Ethical Approval**
Ethical approval for this study was obtained from the Institutional Review Committee (IRC) of the Public Health Concern Trust, Nepal (Phect-Nepal) (IRC No: 043-2017). Written informed consent was obtained from each patient for their voluntary participation in the study. In case of minor(s), written consent was obtained from their parents or guardians. This study was conducted in accordance with the Declaration of Helsinki.

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**Author Contributions**
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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