**Novel Research in Microbiology Journal (2018), 2(5): 65-74**
***Research Article***

**(Print) (ISSN 2537-0286)**
**(Online) (ISSN 2537-0294)**
**https://nrmj.journals.ekb.eg/**
**DOI: 10.21608/NRMJ.2018.17862**

# Detection of *bla*$_{NDM-1}$ gene among the carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from a children's hospital in Nepal

Binod, G.C.[1,2]*; Nim Raj Sapkota[1,2]; Binod Rayamajhee[1,2]; Jayram Lamichhane[1]; Pramod Poudel[1]; Sunil Lekhak[3]; Shovana Thapa[4]; Santosh Khanal[1]

[1]National College, Tribhuvan University, Khusibu, Kathmandu, Nepal; [2]Kathmandu Research Institute for Biological Sciences, Lalitpur, Nepal; [3]Decode Genomics and Research Center, Sinamangal, Kathmandu, Nepal; [4]International Friendship Children's Hospital, Kathmandu, Nepal

* Corresponding author E-mail: gcbinod90@gmail.com

Received: 28 August, 2018; Accepted: 20 September, 2018; Published online: 29 October, 2018

## Abstract

This study was undertaken to detect the presence of *bla*$_{NDM-1}$ gene among the carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates, confirmed through using Modified Hodge Test (MHT). During the six months of this study (November, 2016 – April, 2017), a total of 1503 clinical samples including urine, blood, wound swabs and Cerebrospinal fluid (CSF) were collected and processed at Microbiology Laboratory, International Friendship Children's Hospital, Kathmandu, Nepal. Among the *E. coli* and *K. pneumoniae* isolates, 14.1%, 17.1% were resistant to meropenem, whereas 11.28%, 17.1% were resistant to imipenem, respectively. From the pool of 94 *E. coli* and 35 *K. pneumoniae* isolates, 34 *E. coli* and 18 *K. pneumoniae* were screened as possible carbapenemase producers. For screening of carbapenemase enzyme production among these isolates, resistances to third generation cephalosporins and carbapenems were taken into consideration. Two isolates of *E. coli* and 3 isolates of *K. pneumoniae* were confirmed as carbapenemase producers by MHT. Furthermore, Polymerase chain reaction (PCR) was carried out among the MHT positive bacteria for detection of *bla*$_{NDM-1}$ gene. Genetic analysis of MHT positive isolates showed that 1\2 *E. coli* and 2\3 *K. pneumoniae* isolates were New Delhi metallo-β-lactamase producers (NDM-1). Results of this study demonstrated the presence of *bla*$_{NDM-1}$ gene among the carbapenemase producing *E. coli* and *K. pneumoniae* isolates, and were recorded to be 50% and 66.6%, respectively.

**Key words:** *E. coli*, *K. pneumonia*, *bla*$_{NDM-1}$ gene, Carbapenem resistance, Carbapenemase producers, Enterobacteriaceae

## 1. Introduction

Carbapenems have been used for many years as the antibiotics of choice for treatment of nosocomial infections caused by Enterobacteriaceae. Resistance to these drugs in Enterobacteriaceae has emerged worldwide mostly driven by the production of carbapenemases enzymes particularly *K. pneumoniae* carbapenemase 2 (KPC-2) and more recently (NDM-1) (Padmini and Appalaraju, 2004).

NDM-1 is one of the most important carbapenemases of Carbapenem Resistant Enterobacteriaceae (CRE). Since its first discovery at 2008 in a *K. pneumoniae* isolate recovered from a patient at a hospital, New Delhi, India, it has been transmitted to many species of Enterobacteriaceae in different countries (Yong *et al*., 2009). NDM-1 is most frequently detected in the Indian subcontinent followed by the Balkans region, the Middle East and is mainly associated with community-acquired infections. Yong *et al*., (2009); Deshpande *et al*., (2010) reported that NDM-1 attracted significant attention because the gene encoding this metallo beta lactamase (MBL) is located on a very mobile genetic element, the pattern of its spread proved to be more complex and apparently more unpredictable than the gene encoding *K. pneumoniae* carbapenemase (KPC).

In developing countries like Nepal, many laboratories do not routinely detect carbapenemases production, a practice which likely result in misreporting and hence failure treatments. It is therefore essential that carbapenemase positive rates are monitored, and that decision regarding appropriate laboratory practice must be made in light of local/ regional data of CRE. Moreover, correct reporting would limit misuse of antibiotics and hence decrease emergence and extension of antibiotic resistance worldwide. Presence of NDM-1 has been reported in Nepal along with various types of NDM and other classes of carbapenemases such as KPC. These enzymes make treatment of bacterial infections impossible using carbapenems, thus the only drug left for choice is colistin. The aim of the current study is to defeat this important problem of CRE through focusing the clinical researches toward studying their emergence and spread.

## 2. Materials and methods

### 2.1. Study site and period of study

This study was carried out in Pathology department of International Friendship Children hospital, Maharajgunj, Kathmandu, Nepal, from November, 2016 to April, 2017. During this period, a total of 270 bacterial isolates from 1503 clinical specimens were isolated and processed according to the Clinical and Laboratory Standards Institute Guidelines (CLSI, 2011). Patients under the age of 12 years or their guardians visiting the Pathology department were directly interviewed for his/ her clinical history during samples collection. Furthermore, the tests for genetic analysis of the selected bacterial isolates were carried out in Decode Genomics and Research Center, Sinamangal, Kathmandu, Nepal.

### 2.2. Sample collection and transport

Clinical samples (blood, urine, pus, wound swabs and CSF) were collected and transported to the laboratory following the World health organization (WHO) guidelines. The quality of these samples was evaluated before processing and improper specimens were excluded (Vasanthakumari, 2009).

### 2.3. Isolation and identification of bacteria

Clinical specimens were cultured on selective and differential media such as MacConkey agar and Blood agar, respectively, and then incubated at 37$^{o}$C for 24 h to recover bacterial cultures. Isolated bacteria were identified on the basis of Gram staining and biochemical assays according to (Forbes *et al*., 2007).

### 2.4. Antibiotic susceptibility assay

The antibiotic susceptibility test of Enterobacteriaceae towards various antibiotics were carried out by modified Kirby-Bauer disk diffusion method (Midolo *et al*., 1995), as recommended by CLSI, (2011) on Muller Hilton agar medium. Antibiotics such as Amoxicillin clavunate (20+10 mcg), Amikacin (30 mcg), Cotrimoxazole (25 mcg), Ciprofloxacin (5 mcg), Ceftriaxone (30 mcg), Cefotaxime (30 mcg), Ceftazidime (30 mcg), Gentamicin (10 mcg), Imipenem (10 mcg), Meropenem (10 mcg) and Polymixin B (300 U) were used for antibacterial susceptibility testing and their zones of inhibition (ZI) were detected. ZI of Meropenem and Imipenem were observed for screened bacteria to detect carbapenemase production. Resistance to third generation cephalosporins and carbapenems were recorded as carbapenemase producing Enterobacteriaceae. In reference to Magiorakos *et al*., (2012), bacterial isolates that were resistant or intermediately susceptible to these antibiotics were identified as multidrug resistant Enterobacteriaceae. *E. coli* (ATCC 25922) was used as a control strain.

### 2.5. Phenotypic confirmation of carbapenemase production

The confirmation of carbapenemase production was determined by MHT following Centers for Disease Control and Prevention Guidelines (CDC, 2009). According to these guidelines, a characteristic clover leaf type indentation was expected after 16-24 h incubation of tested bacteria on a MHA plate. A lawn culture of *E. coli* (ATCC 25922) was used as control.

### 2.6. Confirmation of $bla_{\text{NDM-1}}$ gene presence using PCR

Total DNA was extracted from the bacterial isolates by boiling method according to Dashti *et al*., (2009). The PCR mixture for the detection of MBL genes contained 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.125 mM of deoxynucleotide triphosphate, 0.1 μM of each primer), and 2 U of Ampli Taq Gold polymerase (Macrogen, South Korea). The primers assessed in this study were NDM-1 FD (5′-CAACTGGATCAAGCAGGAGA-3′) and NDM-1 RD (5′-TCGATCCCAACGGTGATATT-3′), which amplified an internal fragment of 621 bp of the $bla_{\text{NDM-1}}$ gene (Macrogen, S. Korea). Amplification was carried out under the following thermal cycling conditions: 10 min. at 94°C; 36 cycles of amplification consisting of 30 s at 94°C, 40 s at 56°C, and 50 s at 72°C; and 5 min. at 72°C for the final extension. DNA fragments were visualized by electrophoresis in a 1% agarose gel at 100 V for 1 h in 1× TAE (40 mM Tris-HCl [pH 8.3], 2 mM acetate, 1 mM EDTA) containing 0.05 mg/ l ethidium bromide (Nordmann *et al*, 2011b). *E. coli* ATCC BAA2452 was used as reference positive strain, whereas, *E. coli* ATCC 25922 as negative strain.

## 3. Results

Among the total of 1503 clinical samples, the highest number of samples were blood (71%, n=1065), followed by urine (20%, n=305), pus (5%, n=77), wound swab (3%, n=39), and CSF (1% n=17), respectively.

Out of these samples processed, 270 (17.96%) showed bacterial growth. 151 bacterial cultures were isolated from urine samples, 65 from blood, 41 from pus, 13 from wound swabs, however, no bacterial isolates were recovered from CSF (Table 1).

A total of 212 bacterial isolates belonging to 9 different genera were isolated from the clinical specimens. *E. coli* was the most predominant isolate constituting 94 (63.9%) of all pathogens encountered, followed by *K. pneumoniae* 35 (12.3%) and *Acinetobacter* spp. 17 (7.7%). However, least bacterial count 2 (0.7%) was recorded from each of the *Citrobacter* spp. and *Enterobacter* spp. (Table 2).

**Table 1**: Percentages of bacterial cultures isolated from different clinical samples

| Sample | Growth | No growth | Total | Positivity (%) |
|---|---|---|---|---|
| Urine | 151 | 154 | 305 | 49.5 |
| Blood | 65 | 1000 | 1065 | 6.1 |
| Pus | 41 | 38 | 77 | 53.24 |
| Swab | 13 | 26 | 39 | 50 |
| CSF | 0 | 17 | 17 | 0 |
| Total | 270 | 1239 | 1503 | 17.96 % |

**Table 2**: Bacterial spp. recovered from the clinical samples

| Bacteria | Sample | | | | | Total |
|---|---|---|---|---|---|---|
| | Urine | Pus | Swab | Blood | CSF | |
| *E. coli* | 72 | 13 | 0 | 9 | 0 | 94 |
| *K. pneumoniae* | 18 | 4 | 7 | 6 | 0 | 35 |
| *Acinetobacter* spp. | 8 | 3 | 2 | 4 | 0 | 17 |
| *Pseudomonas* spp. | 4 | 9 | 2 | 0 | 0 | 15 |
| *Proteus* spp. | 8 | 3 | 2 | 0 | 0 | 13 |
| *Citrobacter* spp. | 13 | 0 | 0 | 0 | 0 | 13 |
| *Serratia marcescens* | 12 | 0 | 0 | 0 | 0 | 12 |
| *Enterobacter* spp. | 7 | 0 | 0 | 0 | 0 | 7 |
| *Morganella morganii* | 6 | 0 | 0 | 0 | 0 | 6 |
| **Total** | **148** | **32** | **13** | **19** | **0** | **212** |

Among the 10 antibiotics used in this study, carbapenems were the most effective drugs, where 82.2% of isolates were sensitive to imipenem, 79.1% showed no resistance towards meropenem, however, 100% of isolates were sensitive to Polymixin B. This configuration was followed by other antibiotics such as; amikacin (58.36%), gentamicin (56%), ciprofloxacin (52%) and cotrimoxazole (52%). Isolated Enterobacteriaceae showed least sensitivity towards the β-lactams such as amoxicillin clavunate (46%), and third generation cephalosporins; ceftazidime (36%), ceftriaxone (41.5%) and cefotaxime (44.7%) as clear in (Table 3).

Results showed that total MDR bacteria among the 212 isolates were 133 (46.69 %). This number

was highly dominated by *E. coli* (n=58) followed by *K. pneumoniae* (n=21). MDR percentage was found to be extremely high in *M. morganii*, *Acinetobacter* spp. and *Citrobacter* spp. (Table 4).

**Table 3**: Antibiotics sensitivity of the bacterial isolates

| Antibiotics | Resistant | | Intermediate | | Sensitive | |
|---|---|---|---|---|---|---|
| | % | Number | % | Number | % | Number |
| Amikacin | 41 | 87 | 0.6 | 2 | 58.36 | 123 |
| Gentamicin | 40.8 | 86 | 4.1 | 7 | 55.1 | 119 |
| Ciprofloxacin | 36.1 | 77 | 12.7 | 27 | 51.2 | 108 |
| Cotrimoxazole | 38.01 | 80 | 8.8 | 19 | 53.19 | 113 |
| Ceftazidime | 57.6 | 122 | 6.4 | 14 | 36 | 76 |
| Ceftriaxone | 52.4 | 111 | 6.1 | 13 | 41.5 | 88 |
| Amoxicillin | 49.9 | 106 | 4.7 | 10 | 45.4 | 96 |
| Cefotaxime | 47.6 | 101 | 7.7 | 16 | 44.7 | 95 |
| Imipenem | 10.2 | 21 | 7.6 | 16 | 82.2 | 184 |
| Meropenem | 12.4 | 26 | 8.5 | 18 | 79.1 | 168 |
| Polymixin B | 0 | 0 | 0 | 0 | 100 | 212 |

**Table 4**: Multidrug resistance among bacterial isolates

| **Bacterial isolates** | **Multi Drug Resistance** | | **Total** |
|---|---|---|---|
| | **MDR** | **Non MDR** | |
| *E. coli* | 58 | 36 | 94 |
| *K. pneumoniae* | 21 | 14 | 35 |
| *Acinetobacter* spp. | 10 | 7 | 17 |
| *Pseudomonas spp.* | 7 | 8 | 15 |
| *Proteus* spp. | 3 | 10 | 13 |
| *Citrobacter* spp. | 5 | 8 | 13 |
| *S. marcescens* | 4 | 8 | 12 |
| *Enterobacter* spp. | 2 | 5 | 7 |
| *M. morganii* | 3 | 3 | 6 |
| **Total** | **113** | **99** | **212** |

Within the 94 isolates of *E. coli*, 14.1% were resistant to meropenem and 11.28% resistant to imipenem. Whereas, the resistance pattern in third generation cephalosporins were 50%, 47.8% and 36.6% toward ceftriaxone, cefotaxime and ceftazidime, respectively. 34 *E. coli* isolates were recorded as carbapenem resistant (Table 5).

Among the 35 isolates of *K. pneumoniae*, 17.1% were resistant to meropenem and 17.1% to imipenem. Meanwhile, the resistance to cephalosporins was 48.5%, 54.2% and 42.8% in ceftriaxone, cefotaxime and ceftazidime, respectively. 18 *K. pneumoniae* isolates were recorded as carbapenem resistant (Table 6).

**Table 5**: Screening of carbapenem resistant *E. coli*

| Antibiotics | Resistant | | Intermediate | | Sensitive | |
|---|---|---|---|---|---|---|
| | % | Number | % | Number | % | Number |
| Ceftriaxone | 50 | 47 | 14.1 | 15 | 35.9 | 62 |
| Cefotaxime | 47.8 | 45 | 7.44 | 7 | 44.6 | 42 |
| Ceftazidime | 36.66 | 39 | 7.52 | 8 | 49.82 | 53 |
| Meropenem | 14.1 | 15 | 6.58 | 7 | 73.32 | 78 |
| Imipenem | 11.28 | 12 | 5.64 | 6 | 77.08 | 82 |

**Table 6**: Screening of carbapenem resistant *K. pneumoniae*

| **Antibiotics** | **Resistant** | | **Intermediate** | | **Sensitive** | |
|---|---|---|---|---|---|---|
| | **%** | **Number** | **%** | **Number** | **%** | **Number** |
| Ceftriaxone | 48.5 | 17 | 8.5 | 3 | 42.8 | 15 |
| Cefotaxime | 54.2 | 19 | 11.4 | 4 | 34.4 | 12 |
| Ceftazidime | 42.8 | 15 | 8.5 | 3 | 48.7 | 17 |
| Meropenem | 17.1 | 6 | 5.7 | 2 | 77.2 | 27 |
| Imipenem | 17.1 | 6 | 8.5 | 3 | 74.4 | 26 |

Out of 34 *E. coli* isolates subjected to MHT, 2 isolates were found to be positive to this test, while 32 isolates were negative. Similarly, out of 18 *K. pneumoniae*, 3 isolates only were MHT positive. Between the 2 MHT positive *E. coli*, 1 isolate only (50%) was found to have $bla_{\text{NDM-1}}$ gene, whereas, 2 among the 3 MHT positive isolates of *K. pneumoniae* (66.6%) possess $bla_{\text{NDM-1}}$ gene thus were confirmed to be NDM-1 producers.

## 4. Discussion

Among the 5 different clinical specimens, isolated bacteria were found to be significantly highest in number in urine samples (151/305),

conversely no bacterial growth were encountered in CSF. These results were in agreement with those of Baral, (2008); Poudel, (2010). Presence of fastidious microbes which were not easily grown in routine culture media and pre-antibiotic treatment could be the main factors that caused decrease in the sensitivity of culture media.

*E. coli* was the predominant bacterial isolate recovered. Out of the 212 isolates of Enterobacteriaceae, *E. coli* accounted for 94 (44%) followed by *K. pneumoniae* 35 (16.50%). Greater prevalence of *E. coli* in these clinical specimens more than any other species is not surprising as it is a normal flora of human body, and is highly opportunistic in immunocompromised patients. *E. coli* becomes pathogenic when it reaches the tissues outside of its normal intestinal tract or other less common normal sites. Pathogenic *E. coli* strains encode a number of virulence factors which enable them to colonize the human body and persist in face of highly effective host defense (Bien *et al*., 2012). *E. coli* remains one of the most diverse bacterial species as only 20% of its genome is common to all other strains, and about 2% of *E. coli* DNA consists of mobile genetic elements including; phases, plasmids and transposons. According to Lukjancenko *et al*., (2010), these mobile genetic elements are responsible for the continuous evolution of the bacterial genomic repertoire, thus providing significant diversity in *E. coli* strains. Dobrindt *et al*., (2010) added that pathogenic *E. coli* have evolved from non-pathogenic strains by acquiring new virulence factors through the horizontal transfer of accessory DNA, which is often organized in clusters (39 pathogenicity islands) in the chromosome or on plasmids.

Antimicrobial resistance is a global public health issue, and is now generally accepted as a major significant implication in health and patients care. Resistance to antimicrobial drugs caused increased morbidity and mortality from infectious diseases. Pathogens showed gradual increase of resistance to commonly used antimicrobials such as β-lactams, trimethoprim/ sulfamethoxazole, fluoroquinolones, etc. (Gales *et al*., 2011). Growing rate of resistance to the antibiotic carbapenem is emerging as a great threat in recent years. In the current study within the carbapenems, 82.2% of the isolates were sensitive to imipenem, while only 79.1% were sensitive to meropenem. In aminoglycosides, 58.36% of the isolates were sensitive towards amikacin, whereas, 55.1% were sensitive towards gentamicin. The configuration in the third generation cephalosporins and β-lactams such as amoxicillin plus clavunate were found to be the least among all drugs (45.4%). Among the cephalosporins; cefotaxime was the most effective drug with sensitivity index of 44.7% followed by ceftriaxone (41.5%), and then ceftazidime (36%).

Among the 212 isolates of Enterobacteriaceae, 113 (53.3%) were recorded to be MDR, highest number of MDR however was contributed by *E. coli* (58/94) and *K. pneumoniae* (21/35). These results were in accordance with those of Pokhrel *et al*., (2006); Khanal *et al*., (2013), which indicated the slight increase in level of MDR bacteria in Nepal. MDR bacteria may accumulate multiple genes each coding for resistance to a single drug within a single cell, accumulation occurs typically in resistance (R) plasmids. Moreover, MDR may occur by the increased expression of genes that code for multidrug efflux pumps extruding a wide range of drugs (Nikaido, 2009).

From the pool of 94 *E. coli* and 35 *K. pneumoniae* isolates, 34 *E. coli* and 18 *K. pneumoniae* were resistant to at least one cephalosporin. In this study, imipenem or meropenem were selected as indicative of carbapenemase production or as possible carbapenem resistant bacteria. In similar earlier studies of Zhanel *et al*., (1998); Piller *et al*., (2008), results demonstrated 4-16 folds greater potency of meropenem than imipenem even in *E. coli* and other members of Enterobacteriaceae. On the contrary, in the study of Padmini and Appalaraju, (2004) overall resistance was reported to be 22.2% toward

meropenem and 17.3% to imipenem signifying more effectiveness of imipenem. A report of Rhomborg and Jones, (2009) from United States expressed the significant increase in the meropenem resistant isolates of *K. pneumoniae* from 0.6% to 5.6% in the period of 2004-2008. Antibiotic resistance is mainly determined by the efficiency of enzymes hydrolyzing the drug and by the number of resistance mechanisms present in these microbes. According to current results, MHT demonstrated that among 34 *E. coli* isolates recorded as carbapenem resistant; 2 isolates only were confirmed as carbapenemase producers, whereas 3 isolates of *K. pneumoniae* were reported as carbapenemase producers out of 18.

CLSI, (2009) recommended MHT as a phenotypic confirmatory assay for detection of carbapenemase production in Enterobacteriaceae isolates, with elevated minimum inhibitory concentrations (MICs) for carbapenems or reduced (ZI) in disc diffusion susceptibility testing. This was attributed to acceptable sensitivity and specificity of MHT for carbapenemase detection.

Two isolates of *E. coli* and 3 of *K. pneumoniae* were screened for $bla_{\text{NDM-1}}$ gene, 1 isolate of *E. coli* and 2 of *K. pneumoniae* were confirmed to be NDM-1 producers. In a similar study carried out in India by Datta *et al*., (2012), the prevalence of carbapenem resistant bacteria belonging to Enterobacteriaceae was 7.87%, while the rate of MBL type carbapenemase production was 5.75%. According to an earlier study of Wattal *et al*., (2010), the prevalence of carbapenem resistance in *E. coli* and *Klebsiella* spp. isolated from Intensive care units (ICUs) of a tertiary care hospital in Delhi ranged from 13% to 51%.

Molecular detection of carbapenemase genes is an interesting alternative but remains costly and requires substantial expertise. A novel NDM-13 was identified in a carbapenem resistant *E. coli* clinical isolate obtained from the urine of a patient in Nepal. The enzymatic activity of this NDM-13 against β-lactams was similar to that of NDM-1 (Shrestha *et al*., 2015). Moreover, a new NDM-8 was identified in a multidrug-resistant *E. coli* isolate (NCGM37) obtained from the respiratory tract of a patient in Nepal (Tada *et al*., 2013). Results recorded in the current study and other studies suggested the spread of prevailing NDM-1 as well as novel variants in hospital settings of Nepal.

## Conclusion

*E. coli* and *K. pneumoniae* isolates were found to be the producers of carbapenemase enzyme which is one of the potent factors responsible for resistance of bacteria to carbapenems. Moreover, according to current results; presence of $bla_{\text{NDM-1}}$ gene is one of the significant reasons for growing resistance of Enterobacteriaceae towards carbapenems.

## Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this article.

## Acknowledgements

We gratefully acknowledge the support from the pathology department of International Friendship Children's Hospital (IFCH) Maharajgunj, Kathmandu, and Decode Genomics and Research Centre (DGRC) Sinamangal, Kathmandu, Nepal.

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