Detection of \textit{bla}_{\text{NDM-1}} gene in ESBL producing \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} isolated from urine samples

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Abstract

Introduction: Enterobacteriaceae such as \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} are the most prominent bacterial species resistant to almost all commonly used antibiotics. Carbapenem is one of the last resort drugs for treating such emerging multidrug-resistant bacteria. This study aimed to detect carbapenem-resistant \textit{bla}_{\text{NDM-1}} gene in ESBL producing \textit{E. coli} and \textit{K. pneumoniae} isolates.

Methodology: A total of 190 \textit{E. coli} and 350 \textit{K. pneumoniae} isolates were screened for extended spectrum \textit{β}-lactamase (ESBL), carbapenemase and metallo \textit{β}-lactamase (MBL) production via double-disk synergy test (DDST), modified Hodge test and combined-disk diffusion method. The \textit{bla}_{\text{NDM-1}} gene was detected by PCR and confirmed via Sanger sequencing method.

Results: Of the 540 isolates tested, 71.8\% were found to be multidrug-resistant. Overall rate of ESBL-positive isolates were 57.89\% \textit{E. coli} and 31.42\% \textit{K. pneumoniae}. Among ESBL positive isolates, 49.09\% \textit{E. coli} and 40\% \textit{K. pneumoniae} were positive for carbapenemase production whereas MBL production was detected in 29\% \textit{E. coli} and 22\% \textit{K. pneumoniae} isolates. In MBL positive isolates, (37\%) \textit{E. coli} and (40\%) \textit{K. pneumoniae} isolates harboured \textit{bla}_{\text{NDM-1}} gene. The pair-wise DNA was aligned with the NDM-1 sequence from GenBank. The alignment score was 243 and the blast nucleotide sequencing results showed 97\% sequence similarity with the sequences in GenBank for the \textit{bla}_{\text{NDM-1}} gene.

Conclusions: The \textit{bla}_{\text{NDM-1}} gene was found to be the most prevalent in urine samples. There is a dire need to conduct screening tests in hospitals and communities to find out the exact prevalence of the \textit{bla}_{\text{NDM-1}} spread in our population.

Key words: \textit{bla}_{\text{NDM-1}}; carbapenemase; extended-spectrum-\textit{β}-lactamase; metallo-\textit{β}-lactamase; \textit{E. coli}; \textit{K. pneumoniae}.

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Introduction

New Delhi Metallo-\textit{β}-lactamase (NDM-1) type metallo-\textit{β}-lactamase (MBL) is one of the most potent and prevalent enzymes of MBL in \textit{Escherichia coli}, \textit{Klebsiella pneumoniae} and other Gram-negative rods [1]. The \textit{bla}_{\text{NDM-1}} gene was first detected in 2009 in a Swedish patient, who acquired this gene in India by urinary tract infection, the causative agent of which was carbapenem resistant \textit{K. pneumoniae} [2]. At first, the gene was identified in a 180kb plasmid [3,4], but later on, it was discovered that other plasmids ranged from 50-500 kb of various Gram-negative species, also harbored this gene [5]. This gene is usually present on plasmid and has the capability of transferring from one bacterium to another bacterium, from man to man and even from country to country. Poor sanitation and misuse of antibiotics provokes the spread of NDM-1 [6].

The \textit{bla}_{\text{NDM-1}} gene is present on various plasmids, also containing other resistant genes such as ESBL genes (CTX-M-15, SHV-12), carbapenem-resistant genes or carbapenemase-encoding genes (OXA-48, OXA-181, VIM), plasmid-associated cephalosporinase genes (CMY-16, CMY-58), aminoglycoside resistance genes (16s rRNA methylase), macrolide resistance genes (esterase), rifampin resistance gene, \textit{qnr} genes (\textit{qnrAB}, \textit{qnrB1}, \textit{qnrB2}) and sulfamethoxazole resistance genes, reported from other parts of the world including Pakistan [6–9].

Carbapenem antibiotics, having a large spectrum of activity, were once considered a good option against ESBL producers but due to the emergence of resistance towards carbapenems in the form of carbapenem
hydrolyzing β-lactamases, has reduce the efficiency of carbapenems [10]. This resistance can be acquired by alteration in PBPs which is a target site for carbapenem attachment, alteration in the structure of porin proteins, carbapenem hydrolyzing β-lactamases or by increased action of efflux pumps. Several genes are also responsible for carbapenemase production, the New Delhi metallo β-lactamase-1 (blaNDM-1) being one of them [11].

Carbapenem-resistant genes are rapidly increasing, and over the last few years, carbapenem resistance has become a major health issue. The high prevalence of these genes in Pakistan is due to the inappropriate use of drugs in hospitals and health care settings both public and private. Previous studies show that carbapenemase producing Gram-negative bacteria are prevalent but the magnitude of such infections has not been recorded in Peshawar, Pakistan. Whereas, a study conducted in Islamabad showed that the prevalence of this resistance is 36.7% for NDM-1 among Gram-negative bacteria [8]. The aim of the present study was to determine the prevalence and distribution of the blaNDM-1 gene in E. coli and K. pneumoniae isolates collected from tertiary care hospitals of Peshawar and Islamabad, Pakistan.

Methodology

Study design and sampling

This cross-sectional study involved four major tertiary care hospitals of Peshawar and Islamabad including Khyber Teaching Hospital (KTH), Combined Military Hospital (CMH), Hayatabad Medical Complex (HMC) and Pakistan Institute of Medical Sciences Islamabad (PIMS). The study was conducted during January 2018 to June 2018. A total of 540 non-duplicated clinical isolates of E. coli (190) and K. pneumoniae (350) were isolated from urine samples using standard microbiological collection techniques.

Bacterial isolates identification

The collected clinical isolates were identified as E. coli and K. pneumoniae based on morphological characteristics of bacterial colonies on Cysteine Lactose Electrolyte Deficient media (CLED) and Eosin Methylene Blue (EMB) agar media (Oxoid, UK). Further confirmation of the isolates was done by Gram staining and routine biochemical analysis such as indole test, oxidase test, urease test, citrate test and triple sugar iron test [12]. The identified isolates were stored at -80 °C for molecular detection of resistant genes. Working cultures were maintained on nutrient agar at 8 °C for up to four weeks.

Antimicrobial susceptibility testing

The susceptibility testing for a range of antibiotics was done by Kirby-Baur disk diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [13]. The antibiotics used were ampicillin (AMC = 30 μg), ceftazidime (CAZ = 30 μg), cefixime (CFM = 5 μg), imipenem (IPM = 10 μg), ciprofloxacin (CIP = 5 μg), amikacin (AMK = 30 μg), ceftriaxone (CRO = 30 μg), nalidixic acid (NAL = 30 μg), amoxicillin (AMX = 10 μg), cefotaxime (CTX = 30 μg), gentamicin (CN = 30 μg), doripenem (DOR = 30 μg), levofloxacin (LEV = 5 μg), tigecycline (TGC = 15 μg), and nitrofurantoin (NIT = 10 μg). The isolates that showed resistance to more than three antibiotics together with beta-lactams, were processed further.

Detection of extended-spectrum-β-lactamases (ESBL)

ESBL detection was done by double disk synergy test using Muller Hinton Agar (MHA) media (Oxoid, UK), according to CLSI guidelines [13]. The antibiotic disks used were ceftriaxone (CRO 30 μg), cefotaxime (CTX 30 μg), ceftazidime (CAZ 30 μg), cefepime (FEP 30 μg) and amoxicillin+ clavulanic acid (AUG 20 + 10 μg). The agar plates were incubated at 37 °C for 18 hours. An increase of ≥5 mm in the zone diameter of any of the antibiotic disks used towards the center was considered as ESBL positive [14]. For control strains, DDST positive Pseudomonas aeruginosa (ATCC 27853) strain was used as positive control whereas DDST negative Pseudomonas aeruginosa strain as a negative control.

Detection of carbapenemases

Modified Hodge test (MHT) was performed using MHA agar media along with 10 μg Ertapenem disk for the detection of carbapenemases production [15]. After 18 hours of incubation at 37 °C, plates were observed for the appearance of a clover leaf-like indentation at the intersection point between the test organism and the indicator organism. The K. pneumoniae (ATCC BAA-1705) MHT positive was used as a positive control whereas K. pneumoniae (DSMZ 9377) MHT negative as a negative control.

Detection of metallo-β-Lactamases

MBLs were identified using combined disk diffusion method on MHA media along with 10 μg of meropenem disk and 0.5 M EDTA as an indicator. This procedure was conducted following a previously published method [16,17]. After 18-24 hours incubation time, the plates were observed for a ≥7 mm increase in the zone of inhibition of the combination disks
(meropenem + EDTA) as compared to meropenem disk alone in order to identify MBL positive isolates. The positive control used was MBL positive Pseudomonas aeruginosa, and the negative control was MBL negative Pseudomonas aeruginosa ATCC 27853 strain.

Molecular identification of blaNDM-1 gene by PCR

The Plasmid DNA was extracted by alkaline lysis method [18]. The quantity and quality of the extracted DNA was determined by using Nanodrop (courtesy of Forman Christian College Biotechnology laboratory) followed by gel electrophoresis to visualize the quality and the expected size of plasmid DNA. Amplification of the DNA was done using specific primers for blaNDM-1 gene, as shown in Table 1. The PCR cycling conditions were: 94ºC for 4 minutes, 35 cycles of 94 ºC for 4 seconds, 55 ºC for 40 seconds and 72 ºC for 60 seconds followed by 72 ºC for 10 minutes.

Plasmid stability assay

The plasmids of blaNDM-1 gene carrying isolates were verified for its permanency. Sub-culturing of the isolates was carried out for six consecutive days on MHA media without meropenem and with meropenem disk at 0.5 µg/mL. After every sub-culturing, phenotypic detection of carbapenem resistance and presence of plasmid DNA on 0.7% agarose gel was observed.

Nucleotide sequence interpretation

The PCR products of two NDM-1 positive isolates (one E. coli and one K. pneumoniae) were sent to Macrogen (Korea), an external expertise provider. The nucleotide sequences and the presumed protein sequences were examined with Basic Local Alignments Search Tool (BLAST) [19].

Results

Clinical bacterial isolates

Out of 540 clinical isolates collected from different hospitals in Peshawar and Islamabad, 190 (35.2%) were identified as E. coli and 350 (64.8%) as K. pneumoniae being the most predominant species. The number and frequency of the isolates collected from different hospitals are shown in Table 2.

Antimicrobial susceptibility

In E. coli isolates, highest resistance was observed towards ceftriaxone (74.7%), followed by ampicillin (74.2%) and cefotaxime (69.4%), whereas imipenem (94.7%), doripenem (88.4%), and nitrofurantoin (86.8%) are found to be the most effective drugs in the present study. In K. pneumoniae isolates, lowest susceptibility rate was observed towards amoxicillin (81.7%), ampicillin (77.7%) and cefixime (71.7%). However, K. pneumoniae isolates showed highest susceptibility towards imipenem (98.2%) and doripenem (90%) respectively (Table 3). The overall percentage of MDR among the 540 bacterial isolates was 71.8%. The MDR percentage was dominated in K. pneumoniae (73.1%) as from E. coli isolates (69.4%).

Prevalence of ESBL, Carbapenemase and MBL producing E. coli and K. pneumoniae

Of the total 540 isolates, 57.89% E. coli and 31.42% K. pneumoniae were ESBL positive with DDST. Among 220 ESBL positive isolates of E. coli and K. pneumoniae, 49.09% E. coli and 40% K. pneumoniae were positive for carbapenemase production. Among 98 MHT positive isolates, 29% E. coli and 22% K. pneumoniae were phenotypically positive for MBL production (Table 4).

PCR results

Out of 26 MBL positive E. coli and K. pneumoniae, 10 were found to harbor the blaNDM-1 gene by PCR amplification. The details and characteristic profiles of NDM-1 positive isolates are given in Table 4 and Table 5.

Plasmid stability assay

The plasmid stability was confirmed by repeated sub-culturing of the resistant isolates of E. coli and K. pneumoniae. The plasmid isolated after every subculture was of the same size on 0.7% agarose gel.

Sequence interpretation

The pairwise DNA was aligned with the NDM-1 sequence taken from NCBI using the Sequence Manipulation Suite, a Pair-wise aligned DNA online software. The alignment score was 243, which showed similarity with NDM-1 gene.

Table 1. Primers for amplification of blaNDM-1 gene.

| Sr. No | Target Gene | Primers | Primer Sequences (5' - 3') | Amplicon Size (bp) | References |
|--------|-------------|---------|---------------------------|-------------------|------------|
| 1      | blaNDM-1    | NDM F*  | GGCGAAGTGCCATTCAACCGGT    | 475bp             | [15]       |
|        |             | NDM R†  | GTCAGGTCGCGTTGCGCAT       |                   |            |

* Forward; † Reverse.
Table 2. Number of *E. coli* and *K. pneumoniae* strains isolated from hospitals.

| Hospitals | *E. coli* | *K. pneumoniae* |
|-----------|-----------|-----------------|
| KTH       | 70        | 136             |
| CMH       | 40        | 46              |
| HMC       | 30        | 64              |
| PIMS      | 50        | 104             |
| Total     | 190       | 350             |

KTH = Khyber Teaching Hospital; CMH = Combined Military Hospital; HMC = Hayatabad Medical Complex; PIMS = Pakistan Institute of Medical Sciences Islamabad.

Table 3. Antibiotic susceptibility pattern of *E. coli* and *K. pneumoniae* isolates.

| Antibiotics   | *E. coli* (n = 190) | *K. pneumoniae* (n = 350) |
|---------------|---------------------|--------------------------|
|               | Susceptible (%)     | Resistant (%)            | Susceptible (%) | Resistant (%) |
| Ampicillin    | 25.7                | 74.2                     | 22.2            | 77.7          |
| Ceftazidime   | 53.6                | 46.3                     | 65.1            | 34.8          |
| Cefixime      | 41.05               | 58.9                     | 28.2            | 71.7          |
| Ciprofloxacin | 52.1                | 47.8                     | 67.4            | 32.54         |
| Amikacin      | 48.4                | 51.5                     | 48              | 52            |
| Ceftriaxone   | 25.2                | 74.7                     | 35.4            | 64.5          |
| Nalidixic acid| 38.9                | 61                       | 30.8            | 69.1          |
| Amoxicillin   | 65.7                | 34.2                     | 18.2            | 81.7          |
| Cefotaxime    | 30.5                | 69.4                     | 46.8            | 53.1          |
| Cefalexin     | 42.1                | 57.8                     | 78.8            | 21.1          |
| Doripenem     | 88.4                | 11.5                     | 90              | 10            |
| Levofoxacin   | 56.8                | 43.1                     | 44.2            | 55.7          |
| Tigecycline   | 40                   | 60                       | 29.4            | 70.5          |
| Nitrofurantoin| 86.8                | 13.1                     | 62.8            | 37.1          |
| Imipenem      | 94.7                | 5.2                      | 98.2            | 1.71          |

Table 4. Prevalence of ESBL, carbapenemase, MBL-producing and blaNDM-1 positive *E. coli* and *K. pneumoniae* isolates.

| Bacterial spp. | ESBL positive/total isolates | MHT positive/ESBL positive isolates | MBL positive/MHT positive isolates | NDM-1 positive/MBL positive isolates |
|----------------|-------------------------------|-------------------------------------|-----------------------------------|--------------------------------------|
| *E. coli*      | 110/190 (57.89%)              | 54/110 (49.09%)                     | 16/54 (29%)                       | 6/16 (37%)                          |
| *K. pneumoniae*| 110/350 (31.42%)              | 44/110 (40%)                        | 10/44 (22%)                       | 4/10 (40%)                          |

Table 5. Characteristic profile of NDM-1 positive *E. coli* and *K. pneumoniae* isolates.

| Bacteria | Isolate no | Hospital | ESBL | MHT | MBL | NDM-1 gene |
|----------|------------|----------|------|-----|-----|------------|
| *E. coli*|            |          |      |     |     |            |
| EK10     | KTH        | Positive | Positive | Positive | Present |
| EK14     | KTH        | Positive | Positive | Positive | Present |
| EK33     | KTH        | Positive | Positive | ND | Present |
| EH09     | HMC        | Positive | Positive | Positive | Present |
| EH27     | HMC        | Positive | Positive | ND | Present |
| EP23     | PIMS       | Positive | Positive | Positive | Present |
| *K. pneumoniae* |            |          |      |     |     |            |
| KPK11    | KTH        | Positive | Positive | ND | Present |
| KPH06    | HMC        | Positive | Positive | Positive | Present |
| KPH29    | HMC        | Positive | Positive | Positive | Present |
| KPH55    | HMC        | Positive | Positive | Positive | Present |

KTH = Khyber Teaching Hospital; HMC = Hayatabad Medical Complex; PIMS = Pakistan Institute of Medical Sciences; ESBL = Extended Spectrum beta-lactamase; MHT = Modified Hodge test; MBL = Metallo beta-lactamase; NDM = New Delhi Metallo beta-lactamase; ND = non-determinable.
Furthermore, this sequence was entered in BLAST on NCBI. The blast nucleotide sequencing showed 99% similarity with *E. coli* (accession number MT367572.1) and 98% similarity with *K. pneumoniae* (accession number MT320914.1), containing the NDM-1 gene.

**Nucleotide accession numbers**

The nucleotide sequences of *E. coli* and *K. pneumoniae* isolates carrying *blaNDM-1* gene have been submitted in GenBank under accession numbers MK576132 and MK569761 respectively.

**Discussion**

Regulatory authority’s negligence, poor enforcement efforts and the distribution of antibiotics by poorly trained people are the factors involved in the emergence of antibiotic resistance. The trend of using antibiotics is on rise in many countries [20]. Hence, inappropriate use leads to the selection of resistant bacterial strains that produce infections that are very difficult to treat, resulting in financial burden on health sectors and on society [21].

The emergence of co-resistance of ESBLs along with other β-lactamases is a serious issue worldwide. The *blaNDM-1* gene has already been reported along with other ESBL producing isolates from Pakistan [8]. In the present study we looked for the occurrence of *blaNDM-1* gene in ESBL producing isolates from Peshawar and Islamabad Pakistan.

Here we reported 110 (31.42%) isolates of *K. pneumoniae* as ESBL producers, the same percentage (31.7%) has been reported from India in 2014 [22]. The ESBL ratio in our region is one of the highest ESBL infections reported as compared to Canada (5%), United States (8%), Europe (23%) and Pacific Western region (25%). However, Farhat Ullah *et al.* in 2009 reported this as 54 (58.5%) [23], which is higher as compared to our study. Other studies reported 60.4% ESBL producers in Iran [24] and 50% in Israel [25]. The contradictions among the reported findings could be due to inadequate antibiotic therapy which might have resulted in an increase in the ESBL prevalence [26,27].

The number of ESBL samples of *E. coli* in our study is 110 (57.89%), which is in accordance with the study reported in India as 52.49% [28]. However, the results from Pakistan and India are higher than those observed in Tanzania 15.1% [29], Nepal 9.0% [30], and the Libyan community 13.4% [31]. This wide variation in prevalence is probably due to the type of specimens collected from different.

We report highest percentage of 49.09% of MHT positive *E. coli* isolates and 40% *K. pneumoniae* isolates which varies from other studies such as 30.9% isolates in India in 2011 [32] and 86.8% isolates reported in Madagascar [33]. Romana *et al.* reported 28.7% *K. pneumoniae* and 20.3% *E. coli* as MHT positive isolates [34]. Difference in this ratio could be due to the inclusion of carbapenem resistant isolates only in the above-mentioned studies, while we used clinical isolates specifically from patients with urinary tract infections, followed by checking their resistant pattern.

In our study, 29% *E. coli* and 22% *K. pneumoniae* isolates were reported as MBL producers. China reported 23.3% MBL producing *K. pneumoniae* in 2012 that had significantly increased from 2005 (0.91%) to 2009 (12.8%) [35]. Some studies vary from our study such as a study conducted in 2010 reported 76.8% *E. coli* as MBL producers [36]. Another study in 2011 showed 61.11% *K. pneumoniae* and 57.69% of *E. coli* as MBL producers [37]. Ranjan *et al.* in 2016 showed 8% of *E. coli* as MBL producers [38].

In this study 37% MBL producing *E. coli* and 40% MBL producing *K. pneumoniae* were positive for *blaNDM-1* gene. A study in Pakistan conducted by Nahid *et al.* in 2013 reported 41% *K. pneumoniae* isolates with only 9.0 harboring NDM-1 gene [8]. Recently, a five-year surveillance report of 2010-2014 indicates increased predominance of NDM-1 gene in *K. pneumoniae*, this percentage being 7.1% in 2010, 10.8% in 2011, 39.3% in 2012, 47.5% in 2013 and 63.0% in 2014 [39]. This high increase in the percentages could be due to the difference in sample size or origin of samples.

**Conclusions**

In the present study, the emergence of NDM-1 gene producing *E. coli* and *K. pneumoniae* highlights an urgent need to overcome the over-the-counter availability and the inappropriate use of antibiotics both in public and private health care settings. The drug regularity authority of Pakistan (DRAP) should develop an implementable monitoring and evaluation system to tackle this issue. Besides, alternative therapies should be explored and assessed for treating and preventing carbapenem resistant infections.

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Authors’ Contributions
SN, TUR: study concept and design; SN: acquisition of data; SN, HB: analysis and interpretation of data; SN, MAK, FH: drafting of the manuscript; TUR, HM, SB: critical revision of the manuscript for important intellectual content; TUR: drafting of the manuscript; TUR, HM, SB: critical revision of the manuscript for important intellectual content; TUR: study supervision.

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