New Delhi metallo-β-lactamase - type carbapenemases producing *Escherichia coli* isolates from hospitalized patients: A pilot study

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*Background & objectives:* Resistances to carbapenem group of antimicrobials among *Escherichia coli* due to production of carbapenemases, especially the New Delhi metallo-β-lactamase (NDM) types, pose serious challenges in the treatment of infections in healthcare settings. This study was undertaken to detect NDM producing *E. coli* isolates from hospitalized patients with urinary tract infection (UTI).

*Methods:* A total of 30 non-repetitive isolates of *E. coli* from hospitalized patients with clinical suspicion of UTI were subjected to antimicrobial susceptibility testing. Screening for the production of extended-spectrum β-lactamases (ESBL) was carried out by minimum inhibitory concentration (MIC) test strip ESBL followed by phenotypic confirmation by double-disc synergy test. Phenotypic confirmation of carbapenemase production was carried out by MIC test strip metallo-β-lactamases. Molecular identification of the *blaNDM* gene was carried out by polymerase chain reaction (PCR) and sequencing of the amplified fragment.

*Results:* Seventeen of the 30 isolates were detected as ESBL producers, of which three were found to be carbapenemase producers. NDM genes were detected by PCR followed by gene sequencing in all three isolates positive for ESBL as well as carbapenemase. The amino acid sequence of the three isolates showed complete identity to the reference sequences of NDM-1, NDM-4 and NDM-8, respectively.

*Interpretation & conclusions:* Our study showed the circulation of NDM variants among the clinical isolates of *E. coli* that were producers of ESBL as well as carbapenemase.

*Key words* Extended-spectrum beta-lactamases - metallo-β-lactamase - New Delhi metallo-β-lactamase type 1 - New Delhi metallo-β-lactamase variants

Multiple drug resistance among *Escherichia coli*, a common pathogen associated with urinary tract infection (UTI) in hospitalized patients¹, is regarded as a major problem encountered by clinicians. This is mainly contributed by extended-spectrum β-lactamases (ESBLs) type of resistance among *E. coli* resulting
in resistance to a myriad of antibiotics including third-generation cephalosporin. Carbapenems are powerful group of antimicrobials that are not inactivated by ESBLs and, therefore, are regarded as the treatment of choice for infections by ESBL producers. However, recent reports of resistance to carbapenem due to carbapenemase production have posed serious challenges in the treatment of such infections. The New Delhi metallo-β-lactamase (NDM) and closely related enzymes, which are zinc-requiring metallo-β-lactamases (MBLs), capable of hydrolyzing all penicillins, cephalosporins and carbapenem group of antimicrobials, are among the most recently identified carbapenemases. The gene that encodes NDM is called bla\textsubscript{NDM} gene and has been identified on bacterial chromosomes and plasmids. The present study was carried out to detect and analyze NDM producing isolates of E. coli obtained from hospitalized patients suffering from UTI in a tertiary care hospital in north India.

**Material & Methods**

The study included a total of 30 non-repetitive isolates of E. coli from 66 urine samples randomly selected from the daily urine samples referred from clinically suspected cases of UTI admitted in the Intensive Care Unit of Ram Manohar Lohia Hospital, a tertiary care hospital in New Delhi, India, between May and July 2012. Identification of E. coli isolates was based on culture and biochemical characteristics.

The samples were transported to the Division of Microbiology, National Centre for Diseases Control (NCDC), New Delhi. The study was approved by the NCDC ethics committee.

**Antimicrobial susceptibility testing and determination of minimum inhibitory concentration (MIC) for carbapenems:** Antimicrobial susceptibility testing was carried out by the Kirby-Bauer method and the results were interpreted as per the Clinical Laboratory Standards Institute (CLSI) guidelines. The antimicrobial discs were commercially procured (Becton Dickinson, USA). The antimicrobial discs used were meropenem (MEM-10 µg) and imipenem (IPM-10 µg). In addition, ampicillin (10 µg), co-trimoxazole (25 µg), amikacin (30 µg), gentamicin (10 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), cefoxitin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg) and amoxicillin-clavulanic acid (20/10 µg) discs were also employed. The zone of inhibition was measured and interpreted as per the CLSI guidelines.

Minimum inhibitory concentration (MIC) for carbapenems was determined by commercial MIC test strip containing gradient of antimicrobial concentrations of meropenem and imipenem from 0.002 to 32µg/ml (Liofilchem, Italy; www.liofilchem.net). MIC was determined based on CLSI breakpoint for meropenem and imipenem considering MIC (µg/ml) ≥1 as susceptible, >1 to <4 as intermediate and ≥4 as resistant.

**Screening for ESBL producers:** The screening of all the E. coli isolates for ESBL production was carried on the basis of resistance to cephalosporin by Kirby-Bauer method. These isolates were further screened by MIC test strip ESBL (Liofilchem, Italy) followed by phenotypic confirmation by double-disc synergy test (DDST). DDST was carried out by placing four discs, viz., cefoxitin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg) and cefepime (30 µg), radially at a distance of 20 mm each from a disc containing amoxicillin-clavulanic acid (20/10 µg) on a lawn culture of the E. coli isolate on Mueller-Hinton Agar (MHA) plate. The plates were incubated aerobically at 37°C. The isolates were confirmed as ESBL producer, if the zone size around any of the discs was enhanced towards amoxicillin-clavulanic acid disc.

**K. pneumoniae ATCC 700603 (ESBL producer) strain was used as positive control.**

**Screening for metallo-β-lactamase (MBL) type carbapenemase production:** This was carried out using commercial MIC test strip MBL (Liofilchem), one of the methods commonly employed for presumptive screening of MBL producing strains. The range of antimicrobials in MBL strip had imipenem (IMI) gradient at one end (4-256 µg/ml) and gradient of imipenem (1-64 µg/ml) plus a constant level of EDTA (4 µg/ml) at other end (IMD). The isolate was considered as MBL producer if MIC ratio of (IMI/IMD) was ≥8.

**Detection of New Delhi metallo-β-lactamase (NDM) gene:** The isolates showing evidence of NDM production in MIC test strip MBL were further subjected to detection of bla\textsubscript{NDM} gene by PCR using the pre published sequences, forward 5'-ACCGCCTGGACCGATGACCA-3' and reverse 5'-GCCAAAGTTGGGCGCGGTTG -3' which amplified 264 bp fragment of the bla\textsubscript{NDM} gene. PCR products of all the positive isolates were subjected to sequencing. The amplicons from the positive isolates were purified by PCR purification kit (QIAGEN, Hidden, Germany) and sequenced on ABI PRISM 3130XL sequencer.
(Applied Biosystems, USA) using Big Dye Terminator cycle sequencing kit (Perkin Elmer). In the sequences so obtained, the accuracy of the base calling with the chromatogram peaks was checked using BIOEDIT software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and edited wherever necessary, followed by blast at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast). The derived sequences were aligned with reference sequences from the database of GenBank. The derived sequences were submitted to the GenBank and accession numbers were obtained.

Results
A total of 30 E. coli isolates from 30 UTI patients in Delhi were screened for drug resistance. The age group of these UTI patients ranged from 21 to 70 yr (mean 41±11 yr) with male:female ratio as 1:2.

Overall resistance pattern of E. coli isolates against various antimicrobials were as follows: ampicillin (100%), co-trimoxazole (73.3%), gentamicin (53.3%), amikacin (46.7%), nitrofurantoin (43.3%), norfloxacin (33.3%) amoxicillin-clavulanic acid (43.3%), cefotixin (56.7%), cefotaxime (46.7%), cefazidime (56.7%), cefepime (56.7%), imipenem (10%) and meropenem (10%).

Resistance pattern for carbapenems: Analysis of isolates of E. coli by disc diffusion and MIC test strip determination revealed three of the 30 isolates as resistant to both meropenem and imipenem and additionally three as intermediate to both the antimicrobials.

Screening for ESBL production by MIC test strip ESBL: Of the 30 isolates of E. coli, 17 were phenotypically ESBL producers by MIC test strip method while three were found to be resistant to meropenem and imipenem. However, three more isolates were in the category of intermediate resistant to these two antimicrobials.

Phenotypic confirmation of ESBL production: All the 17 (56.7%) E. coli isolates screened positive for ESBL by disc diffusion and MIC test strip were confirmed to be ESBL producers by DDST. These 17 isolates were resistant to cefepime, thus ruling out AmpC production. AmpC production in the remaining 13 (43.3%) isolates could not be ruled out.

Screening for production of MBL type carbapenemase: Of the six isolates that were either intermediately resistant or resistant to carbapenems (both meropenem and imipenem) by disc diffusion and MIC test strip, only three were further identified as MBL producers by MIC test strip MBL and the other three intermediate resistant isolates did not show any MBL production (Table).

Identification of blaNDM gene: Of the six isolates that were intermediate or resistant to both meropenem and imipenem on the basis of disc diffusion and MIC test strip as well as by MIC test strip MBL, blaNDM gene was detected in the three resistant isolates only that were confirmed as NDM producers by PCR method (Table). This reconfirmed that intermediate isolates were not MBL producers.

Molecular categorization of blaNDM gene: The partial nucleotide sequence of first isolate (NSU_1, Accession number JX292120.1) showed complete identity with the sequence of reference strain NDM-1. Among the remaining two isolates, one (NSU_2, Accession number JX292121.1) showed an amino acid substitution at 154 (Met →Leu) with 100 per cent identity to the NDM-4 reference sequence and the other (NSU_3, Accession

| Isolate number | R/I (by MIC) | MIC meropenem (µg/ml) | MIC imipenem (µg/ml) | MIC test strip ESBL | MIC test strip MBL | PCR for NDM-1 |
|----------------|-------------|-----------------------|----------------------|---------------------|-------------------|---------------|
| STS-1 (NSU_1)  | R           | 6.0                   | 6.0                  | +                   | +                 | +             |
| STS-9 (NSU_2)  | R           | 32.0                  | 32.0                 | +                   | +                 | +             |
| STS-27 (NSU_3) | R           | 24.0                  | 12.0                 | +                   | +                 | +             |
| STS-10          | I           | 2.0                   | 1.5                  | +                   | -                 | -             |
| STS-17          | I           | 3.0                   | 2.0                  | +                   | -                 | -             |
| STS-23          | I           | 2.5                   | 2.0                  | +                   | -                 | -             |
R, resistant; I, intermediate; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; MBL, Metallo-beta-lactamases. +, positive/present; -, negative/absent
number KJ410407) showed mutations at amino acid
130 (Asp→Gly) and at 154 (Met→Leu), identical to
the reference sequence of NDM-8 (Figure).

Discussion
In the present study, ESBL production was seen
in 17 of the 30 E. coli isolates. Multiple surveys have
shown the rate of ESBL production among E. coli
to be highest in India (80%), followed by China (60%), while
rates are lower elsewhere in East and Southeast Asia
(<30%) and other countries such as Europe, Australia
and North America (range 5-10%)12,13. There has been
a steady rise in the prevalence of ESBL producing E. coli in India, the reported prevalence increased from
18 to 40 per cent during 2003 to 2008 while it rose to
40-75 per cent during 2009 to 20122,12-14.

Reports prior to 2006 indicated that most E. coli
isolates were sensitive to carbapenems. Studies carried
out by Akram et al15 (2002 to 2006) and Padmini and
Appalaraju16 (2002 to 2003) in northern India reported
100 per cent susceptibility to imipenem for urinary
isolates of E. coli, while Menon et al17 in their study
from southern India in 2003 reported similar pattern of
susceptibility for imipenem. However, subsequent
reports indicated emergence of carbapenem resistance
among E. coli18. In the present study, three of 30 (10%)
E. coli isolates were resistant to both meropenem and
imipenem. This was in accordance to studies from
elsewhere in India viz., Delhi, Guwahati and Mumbai
reporting resistance from 5.1 to 14 per cent for both
these antimicrobials19-21.

In another study from southern India, of the 4976
samples tested, 74 (1.48%) yielded multidrug resistant
isolates that included 10 E. coli isolates resistant to both
meropenem and imipenem22. In a study from Kashmir,
of the 1625 Gram-negative isolates, 6.0 per cent were
resistant to both meropenem and imipenem23. In a
hospital based study on neonatal septicemia cases from
Kolkata, India, 105 (37%) of the 285 samples yielded
isolates identified as Enterobacteriaceae, including
27 E. coli isolates with the resistance rate for imipenem
as 0 per cent in 2007, 11 per cent in 2008, 50 per cent in
2009, 25 per cent in 2010 and 37.5 per cent in 201124.

Several studies from India reported high
incidence of NDM-like enzyme production among
the carbapenem-resistant E. coli isolates from hospitals. It has been shown that NDM producing
Enterobacteriaceae, including E. coli, are widespread
in India25. The patients presented with a variety of hospital
and community-associated infections, with UTI being
the most common clinical symptom26. Deshpande
et al27 reported NDM-1 in nine E. coli isolates among
24 carbapenem resistant Enterobacteriaceae in a
tertiary care centre25. Of the 74 E. coli isolates showing
resistance to carbapenems, 34 were positive for blaNDM
gene by PCR22. In a study from North-East India on the
incidence of blaNDM gene among the clinical isolates of
E. coli, of the 270 E. coli isolates from various clinical
samples, during 2009-2010, NDM-1 could be detected
in 14 isolates (5.2%)20.

Up till now 12 published new variants of blaNDM
gene differing from each other by one or two residues

![Figure](https://example.com/figure.png)

**Figure.** Molecular categorization of blaNDM gene: The Plot dot identities of the derived sequence from the study (NSU-1, 2 and 3) and published
New Delhi metallo-ß-lactamase-4 (NDM-4) (Accession number: WP032492624) sequence, NDM-8 (Accession number: JQ 348841) sequence
from the database against NDM-1 (Accession number: AHY37787).
have been reported from various countries\textsuperscript{28-31}. However, NDM-1, 4 and 8 producing isolates were found to be circulating in Delhi as shown in our study. The sequence from one isolate (NSU\textsubscript{1}) matched with that of NDM-1. The NDM-4 is known to differ from NDM-1 by a single amino acid substitution at position 154 (Met\textrightarrow{}Leu) and the isolate (NSU\textsubscript{2}) showed 100 per cent nucleotide identity with NDM-4 variant. This amino acid substitution is responsible for an increased hydrolytic activity of NDM-4 compared to NDM-1 towards meropenem and imipenem\textsuperscript{30}. The amino acid sequence of isolate NSU\textsubscript{3} showed substitution at position 130 (Asp\textrightarrow{}Gly) and at 154 (Met\textrightarrow{}Leu) compared with NDM-1 which is identical to NDM-8 in accordance to the published reports\textsuperscript{28-31}.

In a study by Rahman \textit{et al}\textsuperscript{31}, 12.3 per cent (n=13) of isolates belonging to \textit{Enterobacteriaceae} family at a tertiary care hospital in northern India showed resistance or reduced susceptibility to carbapenem (imipenem or meropenem). These isolates were all positive for NDM with 13 isolates as variants of NDM-1 \textit{i.e.} NDM-5(2), NDM-6(8) NDM-7(3)\textsuperscript{31}.

In conclusion, the present study highlighted the continued threat by ESBL producing \textit{E. coli} in the hospital setting. The detection of NDM variants, \textit{i.e.}, NDM-1, NDM-4 and NDM-8 by the \textit{E. coli} isolates warrants further exploration on a large scale in the country to estimate the prevalence of NDM producing strains in the clinical setting and to review future treatment strategies for UTIs in hospitalized patients particularly in the Intensive Care Units. The present study also indicates the importance of regular monitoring of drug resistance in the hospital for an urgent action to be taken for antibiotics stewardship in the country.

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Conflicts of Interest: None.

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