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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 bla_{NDM} 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 patients[1], 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[2]. However, recent reports of resistance to carbapenem due to carbapenemase production have posed serious challenges in the treatment of such infections[3]. 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_{NDM} gene and has been identified on bacterial chromosomes and plasmids[3]. 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[4].

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[5] and the results were interpreted as per the Clinical Laboratory Standards Institute (CLSI) guidelines[6]. 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[6].

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[5]. These isolates were further screened by MIC test strip ESBL (Liofilchem, Italy) followed by phenotypic confirmation by double-disc synergy test (DDST)[7]. 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[7-9]. *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[10]. 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[10].

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_{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_{NDM} gene[11]. 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%), cefoxitin (56.7%), cefotaxime (46.7%), ceftazidime (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 bla_{NDM} 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, bla_{NDM} 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 bla_{NDM} 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

Table. Production of extended spectrum beta-lactamases (ESBL) and New Delhi Metallo-beta-lactamase-1 (NDM-1) among isolates of *Escherichia coli* (n=6) showing resistance or intermediate resistance to carbapenems (meropenem and imipenem)

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

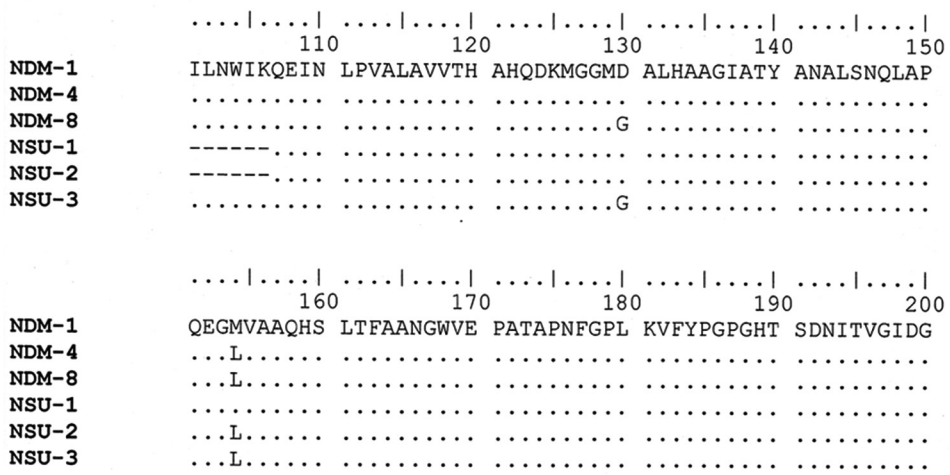

Figure. Molecular categorization of bla_{NDM} gene: The Plot dot identities of the derived sequence from the study (NSU-1, 2 and 3) and published New Delhi metallo-ß-lactamase-4 (NDM-4) (Accession number: WP032492624) sequence, NDM-8 (Accession number: JQ 348841) sequence from the database against NDM-1 (Accession number: AHY37787).

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%)[12-14]. 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 2012[2,12-14].

Reports prior to 2006 indicated that most *E. coli* isolates were sensitive to carbapenems. Studies carried out by Akram *et al*[15] (2002 to 2006) and Padmini and Appalaraju[16] (2002 to 2003) in northern India reported 100 per cent susceptibility to imipenem for urinary isolates of *E. coli*, while Menon *et al*[17] 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. coli*[18]. 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 antimicrobials[19-21].

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 imipenem[22]. In a study from Kashmir, of the 1625 Gram-negative isolates, 6.0 per cent were resistant to both meropenem and imipenem[23]. 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 2011[24].

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 India[25]. The patients presented with a variety of hospital and community-associated infections, with UTI being the most common clinical symptom[26]. Deshpande *et al*[27] reported NDM-1 in nine *E. coli* isolates among 24 carbapenem resistant *Enterobacteriaceae* in a tertiary care centre[25]. Of the 74 *E. coli* isolates showing resistance to carbapenems, 34 were positive for bla_{NDM} gene by PCR[22]. In a study from North-East India on the incidence of bla_{NDM} 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 bla_{NDM} gene differing from each other by one or two residues

have been reported from various countries[28,29]. 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_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→Leu) and the isolate (NSU_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[30]. The amino acid sequence of isolate NSU_3 showed substitution at position 130 (Asp→Gly) and at 154 (Met→Leu) compared with NDM-1 which is identical to NDM-8 in accordance to the published reports[28-31].

In a study by Rahman *et al*[31], 12.3 per cent (n=13) of isolates belonging to *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 *i.e.* NDM-5(2), NDM-6(8) NDM-7(3)[31].

In conclusion, the present study highlighted the continued threat by ESBL producing *E. coli* in the hospital setting. The detection of NDM variants, *i.e.*, NDM-1, NDM-4 and NDM-8 by the *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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