Occurrence of $bla_{NDM}$ Variants Among Enterobacteriaceae From a Neonatal Intensive Care Unit in a Northern India Hospital

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Carbapenem-resistance among enterobacteriaceae has become a global health concern. The objective of this study was to understand NDM producing enterobacteriaceae and their genetic basis of resistance, spreading in neonatal intensive care unit. Carbapenem resistant NDM producing enterobacteriaceae isolates were recovered from rectal swab and blood sample of infants admitted in NICU. These were determined by using Carba-NP test. All isolates were identified using BD Phoenix™−100 and MICs were determined by broth microdilution method. The $bla_{NDM}$ and associated resistant markers were checked by PCR followed by sequencing. Moreover, ERIC-PCR and genetic environment of $bla_{NDM}$ gene were also performed for the analysis of clonal relationship and genetic surrounding of the strains. We characterized 44 isolates with $bla_{NDM}$ variants in $Escherichia coli$ (45.5%), $Klebsiella pneumoniae$ (40.9%), $Citrobacter freundii$ (4.5%), $Citrobacter braakii$ (2.3%), $Klebsiella oxytoca$ (2.3%), $Enterobacter cloacae$ (2.3%), $Enterobacter aerogenes$ (2.2%) from NICU, showing resistance against all antibiotics except colistin and polymixin B. ISABA125 and bleomycin gene were found surrounding all $bla_{NDM}$ variants, besides class I integron on plasmid. (ERIC)-PCR data revealed non-clonal relatedness among most of the isolates. The transfer of resistant markers was confirmed by conjugation experiment. The PCR-based replicon typing was carried out using DNA of transconjugants. These isolates carried NDM-1 (20.45%), NDM-4 (36.36%), NDM-5 (38.64%), NDM-7 (4.55%), along with OXA, CMY, and SHV variants on conjugative plasmid of IncFIA, IncFIC, IncF, IncK, IncFIB, IncB/O, IncHI1, IncP, IncY, IncFIIA, IncI1, and IncN types. An increased number of carbapenem-resistant NDM producing enterobacteriaceae isolates recovered from NICU which is alarming signal for health workers and policy makers. Hence, it is utmost important to think about infection control measures.

Keywords: NDM, carbapenemase, Hospital, NICU, ERIC-PCR, antibiotic resistance, enterobacteriaceae

INTRODUCTION

Emergence of New-Delhi Metallo-β-lactamase (NDM) producers is a matter of concern. The spread of MBL-producing enterobacteriaceae has increased from 2008 onward with the discovery of an ST14 $Klebsiella pneumoniae$ with a new MBL gene, $bla_{NDM}$−1, from a 59-years old Swedish patient who received healthcare in New Delhi, India (Yong et al., 2009). Indian subcontinent are the
most endemic region for the spread of NDM-type MBLs and prevalence rates of NDM-producing enterobacteriaceae were found in range of 5–18.5% in Indian and Pakistan hospitals (Perry et al., 2011; Bharadwaj et al., 2012). In other regions (except the Balkan and Middle East countries), NDM-type MBLs are described mostly as periodic occurrences (Dortet et al., 2014). Carbapenem-resistant microorganisms have become an alarming phenomenon in children (Logan, 2012). A recently published study in USA reported that the frequency of carbapenem resistance increased from 0% in 1999–2000 to 0.47% in 2010–2011 among Enterobacteriaceae isolates in children (Logan et al., 2015). To date, 19 variants of NDM-type carbapenemases (NDM-1 to NDM-19) have been identified (http://www.lahey.org/Studies/other.asp#table1). These variants were identified in expanded species of Gram-negative bacteria and were found to have variation either by multiple residues at different positions or by replacing single amino acid. Recently, an NDM-4, NDM-5, and NDM-7 producing Enterobacter aerogenes from NICU of Indian hospital were reported by our group (Ahmad et al., 2017a). The most widespread variants were found in Indian sub-continent, are NDM-1, NDM-4, NDM-5, NDM-6, and NDM-7 (Khan et al., 2017). Whereas, several types of carbapenemases, such as KPC, IMP, OXA-48, VIM, and New Delhi metallo-β-lactamase (NDM), have been identified globally (Pitout et al., 2015; Logan and Weinstein, 2017).

NDM producing bacteria are resistant to almost all antibiotics, except polymyxins (Kumarasamy et al., 2010). But, the hope of colistin and polymyxins as treatment option has become limited after the discovery of MCR-1 gene in human and animals (Liu et al., 2016). The indiscriminate nature of the gene encoding NDM-1 has made major problem in neonatal intensive care units (NICU). In NICU, high consumption of antimicrobial agents, numerous indwelling devices, and staff rotativity, may further complicate the problem (Zaidi et al., 2005).

In enterobacteriaceae, blaNDM-1 is generally located on conjugative plasmids, ranging from 50 to 200 kb in size and belongs to several incompatibility groups, such as IncL/M, IncH1, IncFII, IncF, or untypable, enabling transfer, and rapid dissemination of multidrug resistance (Poirel et al., 2011).

Our study was designed to evaluate retrospectively the spread of NDM producing Enterobacteriaceae and their genetic basis in neonatal intensive care unit of one of the north Indian tertiary care hospital.

MATERIALS AND METHODS

Collection of Bacterial Strains and Hospital Setting

A total of 750 Enterobacteriaceae clinical isolates were screened from blood and rectal swab of 1,140 neonates admitted in neonatal intensive care unit (NICU) of Jawaharlal Nehru Medical College and Hospital (JNMCH), Aligarh Muslim University, Aligarh, India, during the period, December 2015 to January 2017 in which 308 isolates were found to be carbapenem resistant. It is a tertiary care hospital of 1,300 bed capacity, in which 90 beds were allotted for pediatric patients and 35 beds for the NICU. Patients enrolled in the study were those who enrolled in the active surveillance system (NICU stay 48 h and weekly surveillance swabs taken at least once). Neonates admitted to the ward before December 2015 and/or discharged after January 2017, were excluded.

Ethical Approval

A formal consent from the institutional ethical committee was taken and clearance was obtained from the institute’s ethics committee. Participants/guardians had provided written, informed consent to participate in the study. We have a specific format to get the consents of patients/parents of minors. These formats were made according to the Institutional ethics committee’s guidelines. These forms are confidential and cannot be disclosed as per the guide lines. Institutional ethical committee has already approved. The name of committee/board is “Institutional Ethical Committee of Interdisciplinary Biotechnology Unit [Biot/307/01.06.13],” Aligarh Muslim University, Aligarh, India.

Antimicrobial Susceptibility, Metallo-β-Lactamase (MBL), and MICs Testing

Antimicrobial susceptibility was determined by the standard disc diffusion method using Mueller Hinton agar plate as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016). More than 05 colonies were picked from MH agar plate for antimicrobial susceptibility testing and MBL detection. Detection of metallo-β-lactamase activity was performed, using two imipenem discs (10 μg), one containing 10 μl of 0.1M anhydrous Ethylene Diamine Tetra-Acetic Acid (EDTA). The discs were placed 25 mm distance (center to center) on Mueller-Hinton agar plates (Ahmad et al., 2017b). Minimum Inhibitory Concentrations (MICs) for antimicrobial agents were determined using broth micro dilution method, according to the guidelines of the CLSI.

Carba NP Test for Detection of Carbapenemase

Carba NP test is a biochemical method used for the detection of carbapenemase activity in enterobacteriaceae isolates, performed as described earlier (Nordmann et al., 2012).

Isolate Identification

The species level identification of isolates were performed by using BD Phoenix™-190 automated microbiology system using panel NMIC/ID-55 (Gram negative susceptibility card) and further validated by 16s rRNA sequencing using primer as described previously (Shemesh et al., 2012).

Polymerase Chain Reaction (PCR) Amplification and Sequence Analysis

PCR (Applied Biosystems model-9902 Verity thermo cycler) amplification was performed using primers as described previously (Poirel et al., 2011; Ali et al., 2014) for blaNDM and other resistant marker (blaVIM, blaOXA-1, blaOXA-9, blaCMY, blaTEM, blaSHV, and blaKPC). Amplicons of NDM were purified
from the gel using gel extraction kit (Thermo Fisher Scientific), following manufacturers’ protocol and then sequenced for DNA sequencing at Sci Genom Labs Private Ltd, Cochin, India. The nucleotide and deduced protein sequences were analyzed with software available at the National Centre for Biotechnology Information Website (www.ncbi.nlm.nih.gov).

**Molecular Characterization of Plasmid**

Plasmid DNA extraction and molecular size of multiple plasmids were identified by Kieser method (Kieser, 1984). Plasmid incompatibility group was determined by a PCR-based replicon typing (PBRT) method. Plasmid DNA was amplified by five multiplex and three simplex PCRs using 18 pair of primers as reported previously (Carattoli et al., 2005) that are recognized as Inc. replicon types: FIA, FIB, FIC, HI1, HI2, I1-Ic, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA.

**Conjugation Experiment**

The transfer of resistant markers (blaNDM, blaCMY, blaOXA, and blashv) was determined by conjugation, using an azide-resistant *E. coli* J53 strain as the recipient and isolates as donor (Walsh et al., 2011). Transconjugants were screened on Luria-Bertani agar supplemented with cefazidime (10 µg ml⁻¹) (Sigma-Aldrich) and sodium azide (100 µg ml⁻¹) (HiMedia Laboratories, India). The PCR amplification confirmed the transconjugants having resistant markers.

**Genetic Environment Analysis**

It was performed to identify the genes present at upstream and downstream of blaNDM variants as described previously (Poirel et al., 2011).

**Integron Analysis**

The transconjugants of all the isolates, with blaNDM, were subjected to undergo integron analysis, using PCR amplification of 3’/5’ conserved segment along with Int1 and Sul1 as reported earlier (Dortet et al., 2012).

**Molecular Genotyping of Isolates**

The clonally relatedness between NDM producing isolates were investigated by enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) using the primers ERIC-Forward (5’ATGT AAGCTCCTGGGGATTAAC-3’) and ERIC-Reverse (5’AGTAAAGGACTGGGGTGAGCG-3’), was performed as described earlier (Versalovic et al., 1991). Bio-Red Gel Doc system was used to scan gel image and analyzed the bands by PyElph version 1.4 Software to generate a dendrogram by arithmetic averages (UPGMA) clustering (PyElph) (Pavel and Vasile, 2012).

**RESULTS**

**Isolate Identification**

Of 44 isolates, *Escherichia coli* (*n* = 20; 45.5%), *K. pneumoniae* (*n* = 18; 40.9%), *Citrobacter freundii* (*n* = 2; 4.5%), *Citrobacter braakii* (*n* = 1; 2.3%), *Klebsiella oxytoca* (*n* = 1; 2.3%), *Enterobacter cloacae* (*n* = 1; 2.3%), *E. aerogenes* (*n* = 1; 2.2%), were identified.

**Antimicrobial Susceptibility, Metallo-β-Lactamase (MBL), and MICs**

Of 750 isolates, 44 were found to be New-Delhi Metallo-β-lactamase (NDM) producing enterobacteriaceae strains. All NDM producing strains were found highly resistant antibiotics, including carbapenems (imipenem and meropenem), cephamycin (cefotaxim), extended-spectrum cephalosporins (cefazidime and cefotaxime), aminoglycoside (gentamicin and amikacin), monobactam (aztreonam), tetracycline (minocycline and tigecycline), fluoroquinolone (ciprofloxacin), except polymyxin and colistin. Metallo-β-lactamase (MBL) activity was present in all 44 NDM producing enterobacteriaceae isolates (Table 1). MICs data revealed high values against all tested antibiotics which were found in the range of 128 ≥ 4,096 µg ml⁻¹ (Supplementary Table S1).

**Carbapenemase Production**

All 44 NDM-producing enterobacteriaceae isolates were found positive for Carba-NP test, indicating the production of a carbapenemase as shown in Table 1.

**Detection of Antibiotic Resistance Markers**

PCR amplification and sequencing confirmed that all isolates harbored blaNDM of which NDM-1 (9; 20.45%), NDM-4 (16; 36.36%), NDM-5 (17; 38.64%), and NDM-7 (2; 4.55%) were found to be prevalent. Sequences were submitted to NCBI database (Table 1). Further blaCMY was detected in 20 isolates (8; blacMY-1, 02; blacMY-4, 05; blacMY-145, and 05; blacMY-149) whereas, blaoXA-1 was detected in 37 isolates, and blaoXA-6 was found in 20 isolates. Moreover, 07 blashv-1 and 05 blashv-2 were also found in this study (Table 1). However, blatem, blavim, blaimp, and blakpc were not detected in any of these isolates. Conjugation experiment, further confirmed the presence of these resistance markers on plasmid in each isolate.

**Conjugation**

The plasmidic location of resistant markers was determined by conjugation, using an azide-resistant *E. coli* J53 strain as the recipient [12]. Transconjugants were obtained at the frequencies of 10⁻³ to 10⁻⁵ cells, showing that plasmid from the donors (*E. coli, K. pneumoniae, C. freundii, C. braakii, K. oxytoca, E. cloacae, E. aerogenes*), were found stable in *E. coli* J53.

**Replicon Typing**

These studied NDM producing isolates contained detectable plasmid size (154kb, 66kb, 38kb, 6kb, and 4kb) as shown in Table 1. Number of plasmids were found in the isolates, 1(*n* = 09), 2(*n* = 14), 3(*n* = 15), 4(*n* = 04), 5(*n* = 02). PBRT method identified 12 of 18 replicons types in our study while, IncHI2, IncI1, IncFIA, IncFIB, and IncX were not detected in this study. IncFIA (*n* = 24), IncFIC (*n* = 11), IncF (*n* = 25), IncK (*n* = 36), IncFIB (*n* = 11), IncB/O (*n* = 01), IncH11 (*n* = 01), IncP (*n* = 03), IncY (*n* = 04), IncFIIA (*n* = 16), IncI1 (*n* = 07), and IncN (*n* = 02), replicon types were predominant in the present study and IncFIA, IncFIC, IncF, IncK, and IncFIB were found to be the most frequent types in this study.
| S.No. | Organism name | Isolate Id | Accession no. | NDM variant | Carba NP result | Metallo-β-lactamase | Associated resistance markers* | No. of plasmid/Molecular size in kb* | Plasmid type* | Integron* | Genetic environment of blaNDM |
|-------|---------------|------------|---------------|-------------|-----------------|---------------------|-------------------------------|---------------------------------|--------------|-----------|-------------------------------|
| 1.    | *Escherichia coli* | AK-69      | KX231909      | NDM-7       | Positive        | Present             | OXA-1, CMY-1               | 38, 6, 4                        | FIA, FIC, F, K          | Class 1   | Complete Present               |
| 2.    |               | AK-70      | KX231910      | NDM-5       | Positive        | Present             | OXA-1               | 154, 38, 4                   | FIA, FIC, F, K          | Class 1   | Truncated Present              |
| 3.    |               | AK-71      | KX231911      | NDM-5       | Positive        | Present             | CMY-1               | 66, 38, 4, 6                | FIA, FIC, F, K          | Class 1   | Complete Present               |
| 4.    |               | AK-72      | KX231912      | NDM-5       | Positive        | Present             | OXA-1               | 154, 66, 38, 6             | FIA, FIC, F, K          | Class 1   | Complete Present               |
| 5.    |               | AK-74      | KX231914      | NDM-5       | Positive        | Present             | CMY-149             | 66, 38, 6                   | I, F, K               | Class 1   | Complete Present               |
| 6.    |               | AK-76      | KX231915      | NDM-5       | Positive        | Present             | OXA-1               | 154, 38                     | FIA, F, K              | Class 1   | Complete Present               |
| 7.    |               | AK-77      | KX231916      | NDM-5       | Positive        | Present             | OXA-1, CMY-149      | 66, 38, 6, 4                | FIA, FIB, I, B/O, K     | Class 1   | Complete Present               |
| 8.    |               | AK-79      | KX231918      | NDM-5       | Positive        | Present             | OXA-1, CMY-1       | 38                          | FIA, FIB, F, K          | Class 1   | Complete Present               |
| 9.    |               | AK-80      | KX231919      | NDM-5       | Positive        | Present             | OXA-1               | 38, 2                       | FIA, FIB, F, K          | Class 1   | Complete Present               |
| 10.   |               | AK-81      | KX231920      | NDM-5       | Positive        | Present             | OXA-1, CMY-1       | 38, 6, 4                    | I, F, K               | Class 1   | Truncated Present              |
| 11.   |               | AK-83      | KX231922      | NDM-7       | Positive        | Present             | OXA-1, SHV-1       | 38, 25                      | FIA, FIB, F, K          | Class 1   | Complete Present               |
| 12.   |               | AK-86      | KX231925      | NDM-5       | Positive        | Present             | OXA-1, CMY-1       | 38, 6                       | FIA, F, K              | Class 1   | Complete Present               |
| 13.   |               | AK-87      | KX231926      | NDM-5       | Positive        | Present             | OXA-1               | 38, 6, 4                    | FIA, F, K              | Class 1   | Complete Present               |
| 14.   |               | AK-88      | KX231927      | NDM-5       | Positive        | Present             | OXA-1, OXA-9, CMY-1 | 154, 66                    | FIA, F, K              | Class 1   | Complete Present               |
| 15.   |               | AK-90      | KX231929      | NDM-5       | Positive        | Present             | OXA-1               | 38, 4                       | FIA, F, K              | Class 1   | Complete Present               |
| 16.   |               | AK-91      | KX231930      | NDM-5       | Positive        | Present             | OXA-1               | 154, 66                     | FIA, F, I, K           | Class 1   | Complete Present               |
| 17.   |               | AK-105     | KX099132      | NDM-5       | Positive        | Present             | OXA-1, OXA-9, CMY-1 | 154, 66, 38                | FIA, F, I, K           | Class 1   | Complete Present               |
| 18.   |               | AK-107     | KX099134      | NDM-4       | Positive        | Present             | OXA-1, OXA-9, SHV-1 | 66, 38                      | I, FIA, FIB, F, K      | Class 1   | Complete Present               |
| 19.   |               | AK-109     | KX099136      | NDM-5       | Positive        | Present             | CMY-149             | 38, 6, 4                    | I, F, K               | Class 1   | Complete Present               |
| 20.   |               | AK-116     | KX099143      | NDM-1       | Positive        | Present             | SHV-2               | 154                         | FIA, FIC              | Class 1   | Complete Present               |

(Continued)
| S.No. | Organism name | Isolate Id | Accession no. | NDM variant | Carba NP result | Metallo-β-lactamase | Associated resistance markers* | No. of plasmid/Molecular size in kb* | Plasmid type* | Integron* | Genetic environment of bla<sub>NDM</sub> |
|------|---------------|------------|---------------|-------------|----------------|---------------------|--------------------------------|--------------------------------|--------------|----------|--------------------------------------|
| 28.  | AK-99         | KX999126   | NDM-4         | Positive    | Present        | OXA-1, OXA-9, SHV-2 | 38, 6                         | K, Fila       | Class 1  | Truncated Present                     |
| 29.  | AK-101        | KX999128   | NDM-4         | Positive    | Present        | OXA-1, OXA-9, CMY-145 | 154, 66, 38, 6, 4           | P, FIC, Fila, FIB, F, K Fila | Class 1  | Complete Present                     |
| 30.  | AK-102        | KX999129   | NDM-5         | Positive    | Present        | OXA-1, OXA-9, CMY-4 | 154, 66, 38                 | Fila         | Class 1  | Complete Present                     |
| 31.  | AK-103        | KX999130   | NDM-4         | Positive    | Present        | OXA-1, OXA-9       | 66                           | FIC, K        | ND       | Complete Present                     |
| 32.  | AK-104        | KX999131   | NDM-4         | Positive    | Present        | OXA-1, OXA-9, CMY-4, SHV-1 | 38, 6, 4           | P, FIC, K, Fila | Class 1  | Complete Present                     |
| 33.  | AK-106        | KX999133   | NDM-4         | Positive    | Present        | OXA-1, OXA-9, SHV-2 | 38, 6, 4                     | K            | Class 1  | Complete Present                     |
| 34.  | AK-110        | KX999137   | NDM-4         | Positive    | Present        | OXA-1, OXA-9, CMY-145 | 38, 6, 4                 | K, Fila       | Class 1  | Truncated Present                     |
| 35.  | AK-111        | KX999138   | NDM-4         | Positive    | Present        | OXA-1, OXA-9       | 38, 6, 4                     | K, Fila       | Class 1  | Complete Present                     |
| 36.  | AK-112        | KX999139   | NDM-1         | Positive    | Present        | OXA-1               | 66, 38                       | K             | Class 1  | Complete Present                     |
| 37.  | AK-114        | KX999141   | NDM-4         | Positive    | Present        | OXA-1, OXA-9, SHV-1 | 66, 38                      | K             | Class 1  | Complete Present                     |
| 38.  | AK-115        | KX999142   | NDM-4         | Positive    | Present        | OXA-1, OXA-9       | 38, 6                        | Y, FIA, FIB, F, K Fila | Class 1  | Complete Present                     |
| 39.  | Citrobacter freundii | AK-82 | KX231921 | NDM-4        | Positive    | Present        | OXA-9, SHV-1, CMY-149 | 38             | N, F, K | Class 1  | Complete Present                     |
| 40.  | Citrobacter braakii | AK-113 | KX999140 | NDM-1        | Positive    | Present        | OXA-1, SHV-2, CMY-149 | 66             | FIC, K | Class 1  | Truncated Present                     |
| 41.  | Citrobacter freundii | AK-84 | KX231923 | NDM-4        | Positive    | Present        | OXA-1, CMY-145          | 38             | F      | Class 1  | Complete Present                     |
| 42.  | Klebsiella oxytoca  | AK-100 | KX999127 | NDM-4        | Positive    | Present        | OXA-1, OXA-9            | 154, 66, 38    | I, Y, FIA, F, K Fila | Class 1  | Complete Present                     |
| 43.  | Enterobacter cloacae | AK-108 | KX999135 | NDM-4        | Positive    | Present        | OXA-1, OXA-9, CMY-149   | 66,38          | FIA, FIB | Class 1  | Truncated Present                     |
| 44.  | Enterobacter aerogenes | AK-67 | KX231907 | NDM-1        | Positive    | Present        | OXA-1, SHV-2            | 154, 38, 6, 4  | N, Fila, FIC, K | Class 1  | Truncated Present                     |

*These features were also found on transconjugants.
Integron Analysis
The transconjugants of all isolates harbored plasmid carrying class 1 integron, except two isolates (AK-90 and AK-103) which were confirmed by PCR amplification of 5′/3′ CS, IntI1, and SulI genes. We further confirmed that no resistant marker was present in the integron cassette as shown by a PCR using amplicon of 5′/3′ CS as template.

Genetic Relatedness of the Carbapenem Resistant NDM Producing Enterobacteriaceae Isolates
ERIC-PCR analysis revealed no clonal relatedness among isolates except for the isolates of K. pneumoniae (AK-86 with AK-87, AK-71 with AK-72 and AK-112 with AK-114) as shown in Figure 1.

Genetic Environment of the blaNDM Gene
PCR based genetic environment analysis of blaNDM gene was performed and bleMBL was found at downstream of blaNDM variants in all isolates (Figure 2). A complete ISaba125 sequence was found at upstream of blaNDM in one blaNDM−1 (AK-116), one blaNDM−4 (AK-107), 13 blaNDM−5 (AK-71, AK-72, AK-74, AK-76, AK-77, AK-79, AK-80, AK-86, AK-87, AK-88, AK-90, AK-91, and AK-109), and two NDM-7 producing E. coli (AK-69 and AK-83). Further, complete ISaba125 was amplified in 4 isolates of NDM-1 (AK-66, AK-85, AK-89, and AK-94), eight isolates of NDM-4 (AK-97, AK-101, AK-103 AK-104, AK-106, AK-111, AK-114, and AK-115) and one (AK-102) NDM-5 producing K. pneumoniae (Figure 2). A complete ISaba125 was amplified in three isolates of NDM-4 producing C. freundii, C. braakii, and K. oxytoca, respectively (AK-84, AK-82, and AK-100). However, truncated ISaba125 was detected in three isolates of NDM-5 producing E. coli (AK-70, AK-81, and AK-105). Moreover, 2; NDM-1 (AK-78, AK-112), 3; NDM-4 (AK-98, AK-99, and AK-110), producing K. pneumoniae and one NDM-1 (AK-113) producing C. freundii, one NDM-4 (AK-108) producing E. cloacae and one (AK-67) NDM-1 producing E. aerogenes had truncated ISaba125 at upstream of blaNDM (Table 1, Figure 2).

DISCUSSION
Emergence of NDM-producing enterobacteriaceae has become a globally serious concern. NDM producers led to limited therapeutic options hence it has become a threat to public health. Epidemiological investigation and surveillance of NDMs are of importance to clinical infection control. This study revealed outbreak of multiple variants of blaNDM (9; blaNDM−1, 16; blaNDM−4, 17; blaNDM−5, and 2; blaNDM−7) in clinically important bacteria (20 E. coli, 18 K. pneumoniae, 02 C. freundii, 01 C. braakii, 01 K. oxytoca, 01 E. cloacae, 01 E. aerogenes), as shown in Figure 3.

In E. coli the predominant NDM variant was found to be blaNDM−1, followed by blaNDM−4, blaNDM−5, and blaNDM−7 (Figure 3). Although this is not first description of these NDM
variants being produced by E. coli (Zhang et al., 2013; Qin et al., 2016; Zhu et al., 2016; Pál et al., 2017). Moreover, in these strains existence of NDM and its variants, with CMY, OXA, SHV, and VIM variants and other resistant determinants are documented. Of 20 NDM producing E. coli, one NDM-1 isolate (AK-116) was coexisting with blaSHV−2 and one NDM-4 isolate (AK-107) coexisting with blaOXA−1, blaOXA−9, and blaSHV−1. Further, two isolates of NDM-7 (AK-69, AK-83) were associated with blaOXA−1, blaSHV−1, blaCMY−1, and 16 isolates of blaNDM−5 were linked to blaOXA−1, blaOXA−9, blaSHV−1, blaCMY−1, or blaCMY−4 in different combinations. The most prevalent NDM variants in K. pneumoniae is blaNDM−4, followed by blaNDM−5 and blaNDM−1 (Figure 3). It has also been shown in earlier studies in Klebsiella pneumonia (Khalifa et al., 2016; Petersen-Morfin et al., 2017). Of 18 NDM producing K. pneumoniae, 6 were NDM-1 isolates, coexisting with blaOXA−1, blaOXA−9, blaSHV−1, blaCMY−1, and blaCMY−145. Further, 11 NDM-4 isolates were found associated with blaOXA−1, blaOXA−9, blaSHV−1, blaSHV−2, blaCMY−1, blaCMY−149, and blaOXA−1, blaOXA−9, blaCMY−4 in association with blaNDM−5.

Citrobacter species are rare opportunistic nosocomial pathogens (Ryan and Ray, 2004). It normally causes urinary tract infections, blood stream infections, intra-abdominal sepsis, brain abscesses, pneumonia, and other neonatal infection (Pepperell et al., 2002) such as meningitis, neonatal sepsis, joint infection, or general bacteremia (Doran, 1999). The principal NDM variant found in C. freundii was blaNDM−1 which was followed by blaNDM−4. It is a first report of NDM-4 producing C. freundii (AK-82) co-associated with blaOXA−9, blaSHV−1, and blaCMY−149. Further, C. freundii (AK-113) was also found to have blaOXA−1, blaSHV−2, and blaCMY−149 in association with blaNDM−1.

Moreover, for the first time NDM-4 producing C. braakii (AK-84), K. oxytoca (AK-100), and E. cloaceae (AK-108) were identified in association with blaOXA−1 and blaCMY−145, blaOXA−1 and blaOXA−9 and, blaOXA−1, blaOXA−9, and blaCMY−149, respectively.

We have also identified NDM-1 producing E. aerogenes co-associated with blaOXA−1 and blaSHV−2 in AK-67. NDM-1 producing C. braakii, in Pakistan (Pesesky et al., 2015), NDM-1 producing K. oxytoca in China (Wang et al., 2017), NDM-1 producing E. cloaceae in Turkey (Haciseyitoglu et al., 2017) and Coratia (Petrosillo et al., 2016), have been reported in earlier studies.

The transconjugants were stable and carried all the resistant determinants from donor. Moreover, the presence of class 1 integron in all isolates except AK-90 and AK-103, suggests that the resistant markers can competently exchange among...
Carbapenem resistance among enterobacteriaceae has been considered as one of the most significant menaces to the global healthcare, and the prevalence of NDM variants in enterobacteriaceae has further increased the threat. Therefore, the early detection of the blaNDM possessing enterobacteriaceae isolates with any decreased sensitivity to the carbapenemas is crucial for the choice of the most appropriate antibiotic therapy and the application of efficient infection control measures. The emergence of such resistance patterns may be reduced by the restricted implementation of antibiotics, especially for carbapenems and cephalosporins. Moreover, a strong infection control management in the hospital is necessary to check such infection.

**AUTHOR CONTRIBUTIONS**

NA: performed experiments, wrote draft manuscript; SK: performed experiments; SA: provided samples, and interpreted clinical data; AK: designed study and checked draft manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00407/full#supplementary-material

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Dortet et al., 2014; Jin et al., 2015.

**CONCLUSIONS**

**FIGURE 3** | The clustered bar graph presents the number of NDM variants (each is represented by its own bar) distributed among NDM-producing enterobacteriaceae collected from NICU. The horizontal axis represents the NDM-producing enterobacteriaceae while the vertical axis represents the number of NDM variants.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the authors AK.

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