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Molecular Epidemiology and Genome Dynamics of New Delhi Metallo-β-Lactamase-Producing Extraintestinal Pathogenic *Escherichia coli* Strains from India

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The global dissemination and increasing incidence of carbapenem-resistant, Gram-negative organisms have resulted in acute public health concerns. Here, we present a retrospective multicenter study on molecular characterization of metallo-β-lactamase (MBL)-producing clinical *Escherichia coli* isolates recovered from extraintestinal infections in two hospitals in Pune, India. We screened a large sample size of 510 *E. coli* isolates for MBL production wherein we profiled their molecular determinants, antimicrobial resistance phenotypes, functional virulence properties, genomic features, and transmission dynamics. Approximately 8% of these isolates were MBL producers, the majority of which were of the NDM-1 (69%) type, followed by NDM-5 (19%), NDM-4 (5.5%), and NDM-7 (5.5%). MBL producers were resistant to all antibiotics tested except for colistin, fosfomycin, and chloramphenicol, which were effective to various extents. Plasmids were found to be an effective means of dissemination of NDM genes and other resistance traits. All MBL producers adhered to and invaded bladder epithelial (T24) cells and demonstrated significant serum resistance. Genomic analysis of MBL-producing *E. coli* isolates revealed higher resistance but a moderate virulence gene repertoire. A subset of NDM-1-positive *E. coli* isolates was identified as dominant sequence type 101 (ST101) while two strains belonging to ST167 and ST405 harbored NDM-5. A majority of MBL-producing *E. coli* strains revealed unique genotypes, suggesting that they were clonally unrelated. Overall, the coexistence of virulence and carbapenem resistance in clinical *E. coli* isolates is of serious concern. Moreover, the emergence of NDM-1 among the globally dominant *E. coli* ST101 isolates warrants stringent surveillance and control measures.

Infections with Gram-negative multidrug-resistant (MDR) pathogens, particularly those caused by β-lactamase-producing *Escherichia coli*, constitute a major reason for the "global SOS" in public health (1). Carbapenems are often considered to be the last-resort antibiotics available for treating infections caused by extended-spectrum-β-lactamase (ESBL)- or AmpC-producing bacteria (2) and are categorized in three major types: the KPC types, the metallo-β-lactamases (MBLs), and the oxacillinases (3). The major geographically widespread MBLs consist of imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), and New Delhi metallo-β-lactamase (NDM); NDM has attracted significant worldwide attention as the NDM gene has been detected on different transferable plasmid types (both on typeable and untypeable) and has also been shown to be chromosomally integrated in other bacterial species (4, 5).

NDM belongs to the amber class B β-lactamase. Unlike other classes of β-lactamases (A, C, and D), NDM requires zinc ions to catalyze the hydrolysis of β-lactam antibiotics, and due to this, MBL activity can be inhibited in the presence of metal chelating agents such as EDTA (6). NDM-producing bacteria are mostly multidrug resistant and show resistance toward sulfonamides, fluoroquinolones, aminoglycosides, and macrolides but are susceptible to only a few antimicrobials, such as aztreonam, tigecycline, and colistin (7, 8). Recent reports suggest that this susceptibility is expected to be short-lived (1). Moreover, NDM enzymes are generally not inhibited by any of the known and available β-lactamase inhibitors such as sulbactam, clavulanate, and tazobactam (9).

NDM-producing bacteria have been implicated in both hospital- and community-acquired infections and have been recovered from several infection sites, including those associated with urinary tract infections, pneumonia, septicemia, and wound infections as well as from device-associated infections and companion animals (10–13). Although the exact geographical origin and the precise time for the emergence of the bla_{NDM} gene are unknown, the first NDM-1-positive isolate was recovered in 2009 from a Swedish patient who previously (2007) had an episode of hospitalization in New Delhi, India (14). Later, in the year 2010, Kumarasamy et al. provided evidence that NDM-1-positive *Enterobacteriaceae* are widespread in India, Pakistan, and the United Kingdom (15). Recent reports further indicate that several variants of NDM (NDM-1 to NDM-14)-producing *Enterobacteriaceae* are currently spreading worldwide, including in the United States, Netherlands, Australia, Canada, France, and Oman (16, 17) (http://www.lahey.org/studies). Most of these case reports/epidemiological studies provide evidence of connections of pa-

tients to the Indian subcontinent in the form of migration/travel visits or medical treatment received there. However, certain reports indicate infection sources outside this region with patients without any obvious connection to this area ([18](#)). Nevertheless, the Indian subcontinent particularly offers several risk factors for the emergence and spread of NDM-producing superbugs, which include poor sanitation, easy access to broad-spectrum antibiotics over the counter without proper prescriptions, increased medical tourism for health care, and lack of stringent antibiotic policies.

Members of *Enterobacteriaceae*, particularly *E. coli*, are often disseminated globally through distinct and successful lineages, such as sequence types (ST) 131, 95, 10, etc. ([19–22](#)). Our previous studies have put forward strong evidence for the widespread occurrence of ST131 strains among extraintestinal infections in India and have also reported the existence of NDM-1-positive ST131 *E. coli*, thus representing a serious therapeutic challenge ([23–27](#)). This, together with the implication of NDM-producing strains globally, warrants serious attention of health care specialists and epidemiologists with respect to surveillance and resistance tracking in India and elsewhere so as to monitor threats locally and globally. Also, the data on the virulence traits associated with NDM-producing *E. coli* strains are currently scarce. With the advent of high-resolution, whole-genome sequencing methods, it has now become easy to decipher the genomes of the NDM-producing isolates and understand the underlying resistance and virulence mechanisms. The use of comparative genomics in infection tracking has enabled a comprehensive understanding of epidemiology, resistomes, virulomes, and the genetic background of NDM-producing MDR *E. coli* strains.

In the present study, we sought to determine the prevalence and phenotypes of MBL-producing strains from among a large number of *E. coli* isolates collected chronologically for 6 years (2009 to 2014) from a major tertiary hospital and to determine the antibiotic susceptibility patterns by taking into account the allelic diversity of bla_{NDM} genes and the plasmid replicon types. Further, we determined the virulence traits of NDM-producing *E. coli* by various phenotypic assays in conjunction with whole-genome sequences of five MDR NDM-producing *E. coli* strains and consequent analyses of resistomes, virulomes, and genetic affinities as important parameters required to portray a correct epidemiological scenario. We believe that such exhaustive analyses potentially provide for a clear and solid epidemiological baseline to guide an effective antibiotic policy in a country of endemicity such as India.

MATERIALS AND METHODS

Bacterial isolates. A total of 510 clinical isolates of *E. coli* were included in this study. The isolates were collected consecutively between January 2009 and December 2014 from in-patients and outpatients treated at two major tertiary care hospitals in Pune, India. The collection included clinical isolates from urine ($n = 393$), pus ($n = 88$), and other sources ($n = 29$). All the isolates were obtained as pure cultures and were cryopreserved at $-80°C$ in 20% glycerol. The isolates were revived on LB agar plates and in LB broth prior to experiments. Collection of isolates was in accordance with the guidelines/approvals of Institutional Biosafety Committees at participating institutes/centers, where appropriate.

Detection of metallo-β-lactamase production and its molecular characterization. A total of 510 *E. coli* isolates were screened for MBL production using meropenem (with and without EDTA) with the help of Ezy MIC Strips (HiMedia; India), as per the manufacturer's instructions. All positive *E. coli* strains were also screened for the presence of bla_{NDM-1} by a gene-specific PCR ([28](#)). *E. coli* strains confirmed as MBL producers

were further analyzed by high-fidelity PCR (with the use of Q5 DNA polymerase from NEB) using flanking primers of the NDM gene, as described earlier ([29](#)). Custom sequencing of the resulting amplicons was performed, and the nucleotide and translated protein sequences were analyzed using MEGA6 ([30](#)). NDM types were determined in accordance with the sequences available in the NCBI database(s).

Testing for antimicrobial susceptibility and its molecular determinants. Antimicrobial sensitivity of 39 strains against 23 different antibiotics was performed on Mueller-Hinton agar (MHA) plates by the Kirby-Bauer disc diffusion method using antibiotic discs (HiMedia) and Icosa urinary tract infection (UTI) rings (HiMedia), according to the manufacturer's protocols. Two in-house strains that were consistently resistant and susceptible to most of the antimicrobial agents were employed as positive and negative controls, respectively. Results were interpreted according to the criteria of the Clinical and Laboratory Standards Institute. For colistin, a zone of <11 mm was considered resistant. The MIC values of four frontline antibiotics, namely, tetracycline, co-trimoxazole, gentamicin, and ciprofloxacin, were determined by using HiComb MIC strips (HiMedia, India) as per the manufacturer's protocol. MIC values were determined and compared among a subset of 10 MBL-producing strains, 10 non-ESBL-producing strains, and 7 meropenem-resistant transconjugants. All MBL-producing *E. coli* strains were analyzed for molecular determinants of antibiotic resistance by PCR. DNA template was prepared using a standard heat lysis method. Thereafter, gene-specific primer sets were used to screen for the following ESBL genes: bla_{TEM}, bla_{SHV}, $bla_{CTX-M-15}$, bla_{OXA-48}, bla_{KPC}, and bla_{NDM-1} ([28](#), [31](#)). Other antibiotic resistance genes, such as those conferring resistance to tetracycline [tet(A) and tet(C)], sulfonamides ($sul1$ and $sul2$), streptomycin ($strA$), and trimethoprim (dfr) and including the quinolone resistance determinants (carried on plasmids) such as $aac(6')$-lb, $qnrB$, and $qnrS$, were amplified as per standard PCR protocols ([32](#), [33](#)). The amplicons were run on 1% agarose gels, and the images were documented using a gel documentation system (Major Sciences, USA). The correlation between the phenotypic and different genotypic determinants of resistance was calculated with the help of observed phenotypic resistance and the frequency of the genotypic determinant probed.

Conjugation, mating experiments, and plasmid analysis. Conjugal transfer of resistance genes was performed by broth mating or filter mating experiments for a subset of nine carbapenem-resistant *E. coli* strains using the sodium azide-resistant recipient strain *E. coli* K-12 J53 ([34](#)). Transconjugants were selected on selection plates supplemented with a combination of meropenem (4 μg/ml) and sodium azide (50 μg/ml). In addition, other antibiotics and sodium azide combinations were used to select transconjugants. However, as meropenem-containing transconjugants were the focus of this study, the same were subjected to further analyses. Pure cultures of the transconjugants were preserved in 20% glycerol at $-80°C$. Antibiotic susceptibility and MICs were compared between the parental strains and their respective transconjugants. Plasmid classes were determined using PCR-based replicon typing ([35](#)).

Phenotypic assays. A total of nine random isolates were analyzed for functional virulence traits as described below. Nine MBL-producing *E. coli* strains were tested for their adhesion and invasion efficiency. Human bladder epithelial cell lines (T24) were cultured and maintained in RPMI 1640 medium (Invitrogen, USA) with 10% fetal bovine serum (FBS). Cells were seeded in 24-well plates and grown until a monolayer was obtained. Infection was done at a multiplicity of infection (MOI) of 10. After 3 h of incubation at 37°C with 5% CO_2, cells were washed three times with 1× phosphate-buffered saline (PBS), followed by lysis with 1 ml of 0.1% Triton X-100. Cell lysates were serially diluted, plated on LB agar plates, and incubated overnight at 37°C. For invasion assays, after 3 h of incubation, the cells were washed and further incubated for 1.5 h with 1 ml of RPMI medium containing 100 μg/ml of fosfomycin. After incubation, cells were washed three times with 1× PBS and processed in a way similar to that described above. Assays were performed three times with three technical replicates.

Serum resistance was determined for nine MBL-producing *E. coli* isolates by *in vitro* time-kill experiments as described earlier (22). Briefly, 5 μl of overnight-grown culture was added to 495 μl of LB broth and incubated for 1 h at 37°C in a shaker incubator. The culture was pelleted and resuspended in 1 ml of 1× PBS, and then 30 μl of this bacterial suspension was added to 96-well microtiter plate wells, each containing 270 μl of 50% sterile human serum (Pan Biotech, Germany) in 1× PBS. Thirty microliters of serum was then collected (0-h count), serially diluted, and plated on LB agar plates for CFU enumeration. The plate was then incubated for 3 h at 37°C. After the incubation time (3-h count), 30 μl of sample was pipetted for serial dilution and plated as described above. Growth was measured in terms of CFU counts. Samples were considered serum resistant if the 3-h CFU count equaled or exceeded the 0-h CFU count of the same sample. The serum assay was performed twice in triplicates.

Total siderophore production was tested by streaking all 39 MBL-positive *E. coli* samples on chrome azurol S (CAS) agar plates (blue medium). Color changes (haloes) from yellow to orange on the CAS agar medium plates, after overnight incubation at 37°C, were recorded. No color change in the medium corresponded to no siderophore production by the culture.

ERIC-PCR-based strain typing. Enterobacterial intergenic repetitive element sequence (ERIC)-PCR-based fingerprint analysis was performed as described previously (36). Strain-specific DNA banding profiles obtained on 1.5% agarose gel images were compared using BioNumerics software (version 7.1; Applied Maths, Belgium). After the bands were scored on dice similarity indices based on the unweighted-pair group method using average linkages (UPGMA; arithmetic mean), a dendrogram was generated in order to analyze similarity/diversity within carbapenem-resistant *E. coli* strains. The definition of ERIC clusters was based on a Dice similarity index.

WGS, assembly, annotation, and genomic analysis. Whole-genome sequencing (WGS) of five selected strains was carried out on an Illumina MiSeq system with an insert size of 400 to 500 bp. The paired-end sequence reads were filtered and trimmed using the NGS QC Toolkit (version 2.3.3) (37). These high-quality reads were assembled into contigs using SPAdes Genome Assembler (version 3.6.1) (38). The contigs were then ordered and scaffolded using an in-house workbench, Contig-Layout-Authenticator (39), and the resulting ordered scaffolds were joined by placing Ns between them to construct draft genomes. These were then submitted to the RAST (40) server for annotation, and the genome statistics were extracted using ARTEMIS (41). For all of the genomes, genes were predicted using GeneMarkS (42). Amino acid sequences of the products of the predicted genes from all strains were compared using blastp (43) against the database of *E. coli* virulence genes from the Virulence Factors Database (VFDB) (44). Only genes with 60% identity and 85% query coverage were considered present, and in this way the presence-absence status of each virulence gene in each of these strains was represented in the form of a heat map using R. A similar heat map was generated for putative resistance-related genes after comparison with the Comprehensive Antibiotic Resistance Database (CARD) (45), using blastp. Insertion sequence (IS) elements and putative phage sequences in the genome were identified using IS Finder (46) and PHAST (47), respectively. The sequence type (ST) of each of these strains was determined by submitting the contigs to the server of the Centre for Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk/services/MLST/).

Statistical analysis. All statistical analyses were performed using GraphPad Prism (version 5.01). For adhesion and serum resistance, a nonparametric Mann-Whitney U test was performed while for the invasion assay Wilcoxon matched-pair tests were performed, and *P* values were determined.
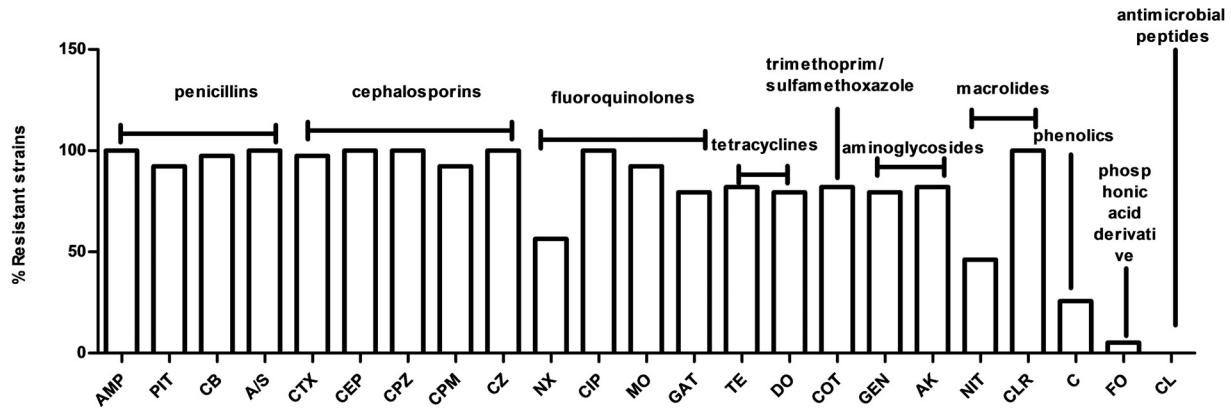
Accession number(s). The results of this whole-genome shotgun project have been deposited in the DDBJ/ENA/GenBank databases under the accession numbers LXFJ00000000 (NA703), LXFK00000000 (NA724), LXEZ00000000 (NA086), LXFA00000000 (NA099), and LWTZ00000000 (NA084). The versions described in this paper are LXFJ01000000, LXFK01000000, LXEZ01000000, LXFA01000000, and LWTZ01000000, respectively.

RESULTS

Prevalence of MBL producers was low with a high abundance of the NDM-1 genotype. Of the 510 clinical *E. coli* isolates, 39 (7.6%) were found to be MBL producers. These 39 MBL-producing *E. coli* isolates were deemed to be of potential interest and were used further for in depth analyses, as described above. All of the 39 MBL-producing *E. coli* isolates were also found to be ESBL producers phenotypically. Most of the MBL producers (25/39) were from urine isolates, followed by those from pus (11/39) and other sources (3/39). The 39 MBL producers as identified at the phenotypic level were screened for the presence of major carbapenemase genes, including bla_{NDM}, bla_{KPC}, and bla_{OXA-48}. With the exception of the bla_{KPC} gene, which was not detected in any of the MBL producers, 36 (92%) MBL producers were bla_{NDM} positive, and 6 (15%) of them were also bla_{OXA-48} positive. Sequence analysis of the full-length NDM genes from 36 MBL producers revealed that the majority of them carried the bla_{NDM-1} gene (25 isolates), followed by bla_{NDM-5} (7 isolates). Further, the gene bla_{NDM-4} was present in two isolates, and the gene bla_{NDM-7} was harbored by two other MBL producers.

All MBL-producing *E. coli* isolates were identified to be MDR and pan-drug resistant. Of the 23 antimicrobial agents representing 10 different classes, our MBL-producing *E. coli* isolates showed an average resistance to nearly 18 antibiotics, with maximum resistance to 21 and minimum resistance to 10 antimicrobial agents. The frequencies of susceptibility/resistance are indicated in Fig. 1. Among all MBL-positive isolates, highest resistance was observed against the β-lactam antibiotic class while highest sensitivity (100%) was observed to the peptide antibiotic class, i.e., colistin. Each of the MBL-producing *E. coli* isolates was found to be at least 90% resistant to all the β-lactam and β-lactam/β-lactamase inhibitor combinations used, including ampicillin, carbenicillin, cefotaxime, cephalothin, cefoperazone, cefepime, cefazolin, piperacillin-tazobactam, and ampicillin-sulbactam. In contrast, each of the MBL-producing *E. coli* isolates was found to be sensitive to colistin. All MBL-positive isolates were highly susceptible to fosfomycin and chloramphenicol (Fig. 1). Other resistance patterns were as follows: 80% of isolates were resistant to each of the tetracycline and aminoglycoside classes and 82% were resistant to the trimethoprim/sulfonamide class. Notably, all MBL-positive isolates (100%) were identified to be MDR as they were resistant to three or more classes of antimicrobials. Moreover, all the MBL-producing *E. coli* isolates were found to be pan-drug resistant, meaning that they were resistant to as many as seven antimicrobial agents. The MIC values of MBL-ESBL-producing *E. coli* and non-ESBL *E. coli* isolates varied widely for different antibiotics used in the study (Table 1). Overall, the MBL-ESBL-producing *E. coli* isolates revealed higher MICs of all four frontline antimicrobial substances than non-ESBL strains. That the MIC values of the four antimicrobials for the meropenem transconjugants were higher than those of the recipient strain J53 *E. coli* indicated horizontal transmission of respective resistance genes. All 39 ESBL-MBL producers were negative for bla_{SHV} and bla_{KPC}. However, 31 isolates harbored the $bla_{CTX-M-15}$ gene, and 28 isolates harbored bla_{TEM}. The correlation among different resistance genotypes (presence or absence of resistance genes) and phenotypes (resistance or susceptibility) was found to be high for aminoglycosides (94%

FIG 1 (Top) Antibiogram of MBL-producing carbapenem-resistant *E. coli* strains as tested by the disc diffusion method for the following 23 different antibiotics: ampicillin (Amp), piperacillin-tazobactam (PIT), carbenicillin (CB), ampicillin/sulbactam (A/S), cefotaxime (CTX), cephalothin (CEP), cefoperazone (CPZ), cefepime (CPM), cefazolin (CZ), nalidixic acid (NX), ciprofloxacin (CIP), moxifloxacin (MO), gatifloxacin (GAT), tetracycline (TE), doxycycline HCl (DO), co-trimoxazole (COT), gentamicin (GEN), amikacin (AK), nitrofurantoin (NIT), clarithromycin (CLR), chloramphenicol (C), fosfomycin (FO), and colistin (CL). All MBL-producing isolates were detected to be pan-drug resistant. (Bottom) Identification of resistance determinants among 39 MBL-producing *E. coli* isolates by gene-specific PCRs.

agreement) and sulfonamides (84% agreement), whereas the agreement between resistance to tetracycline and the presence of *tet*(A) and *tet*(C) was 69% and that for fluoroquinolone was (67%). The overall prevalences of all the major resistance genes in 39 MBL-producing *E. coli* isolates are presented in Fig. 1.

Metallo-β-lactamase resistance was naturally transferable via plasmids. NDM genes are basically located on plasmids. In order to determine the capability of these MBL-positive *E. coli* strains to disseminate NDM genes horizontally, we carried out conjugation mating experiments with the sodium azide-resistant recipient strain *E. coli* K-12 J53. Out of the nine representative MBL-positive strains employed, we were able to obtain seven meropenem-resistant transconjugants alongside several other transconjugants. However, only meropenem transconjugants were further analyzed for this study. The transconjugants were analyzed for their phenotypic drug resistance to four different classes of antibiotics, along with MBL and ESBL detection. All transconjugants were identified to be MBL and ESBL positive, with coresistance varying widely for different antibiotics used in the study. The detailed results are shown in Fig. 2. Transconjugants were confirmed to be carrying the NDM gene by gene-specific PCR. PCR-based replicon typing revealed the presence of primarily two plasmid types, the FIA and FIB plasmids, among transconjugants. These observations reflect the role of transmissible plasmids in dissemination of NDM-based carbapenem resistance.

MBL-producing *E. coli* isolates were moderately virulent. Phenotypic virulence properties of the MBL-positive *E. coli* iso-

lates were assayed using adhesion, invasion, serum bactericidal, and siderophore production assays. The two former assays were carried out on nine randomly selected strains, and siderophore production was determined for all 39 strains. Adhesive and invasive virulence properties are important features during intestinal as well as extraintestinal infections. In order to evaluate this, we quantitatively assayed the pathogen's ability to adhere and invade the urinary bladder epithelial cells. Compared to the negative-control strain *E. coli* DH5α, all nine MBL-positive *E. coli* isolates showed significant adhesion to T24 cells with various efficiencies, as illustrated in Fig. 3a. Among the nine MBL-positive isolates tested, the NA084 isolate demonstrated highest adherence capability toward T24 cells. T24 cells were invaded by all investigated isolates, in contrast to the absence of invasion by the negative-control strain *E. coli* DH5α. Numbers of intracellular bacteria are depicted in Fig. 3b.

Resistance to serum is quite essential for the pathogens to survive and cause invasive infections; we found all nine tested isolates to be resistant (100%) to serum bactericidal activity compared to results with the control DH5α *E. coli*. Bacterial survival in serum over a period of 3 h is depicted in Fig. 3c. Though the virulence genes tested were not prevalent in these isolates, bacterial resistance toward the bactericidal effect of serum could result from individual or combined effects of capsular polysaccharide, surface proteins, and toxin secretions.

Siderophore production, as visualized by an iron mobilization zone around the bacterial colonies with orange haloes on the chrome azurol S agar, was similarly prevalent among 36 (92%) of

TABLE 1 MIC values of carbapenem-resistant strains, meropenem transconjugants, a recipient strain, and non-ESBL non-carbapenem-resistant *E. coli* isolates from the present study

Strain group and no.	MIC (µg/ml)[b]			
	Gentamicin	Ciprofloxacin	Co-trimoxazole	Tetracycline
Carbapenem-resistant strains ($n = 17$)[a]				
NA084	>240	>240	>240	3
NA644	>240	>240	>240	5
NA708	>240	60	>240	3
NA086	>240	>240	>240	2
NA086 T	>240	0.01	0.05	0.1
NA099	>240	>240	>240	0.1
NA099T	0.01	0.1	0.01	0.01
NA316	>240	>240	>240	3
NA316T	0.1	ND	0.05	0.1
NA635	>240	120	>240	5
NA635T	24	ND	>240	3
NA703	>240	>240	>240	0.001
NA703T	16	ND	0.05	0.001
NA724	>240	120	>240	3
NA724T	30	0.01	0.05	0.1
NA805	60	10	>240	0.01
NA805T	ND	1	0.05	0.01
Recipient strain ($n = 1$)				
K-12 J53	0.001	0.008	0.05	0.01
Non-ESBL non-carbapenem-resistant strains ($n = 10$)				
NA433	0.1	60	>240	10
NA010	0.1	0.008	30	0.1
NA295	0.01	0.01	120	0.1
NA053	0.25	1	>240	2
NA057	0.1	30	>240	0.1
NA120	0.01	0.1	>240	0.5
NA140	0.1	0.01	>240	30
NA150	0.1	0.25	2	0.01
NA203	0.01	0.01	0.1	0.01
NA274	0.1	10	>240	30

[a] The total includes seven meropenem transconjugants (T suffix).
[b] ND, not determined.

the 39 MBL-producing *E. coli* isolates. The role of iron in the virulence mechanism of human and animal pathogens is well established (48). Siderophore production has been shown to be more frequently associated with extraintestinal pathogenic *E. coli* (ExPEC).

Whole-genome analysis reveals high resistance and moderate virulence of MBL strains. Out of the 39 MBL-positive strains, whole-genome sequencing of five representative strains (three NDM-1 and two NDM-5) was carried out. Their genome charac-
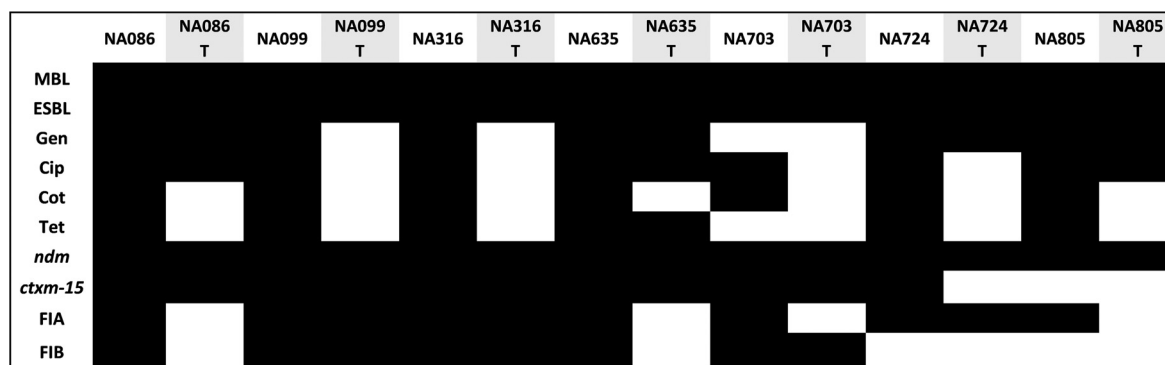

FIG 2 *In vitro* conjugation experiment and comparative analysis between clinical strains and their respective transconjugants revealed transfer of resistances via plasmids. The T suffix designates transconjugants. Gen, gentamicin; Cip, ciprofloxacin; Cot, co-trimoxazole; Tet, tetracycline. *ndm* and *ctxm-15* represent *bla*$_{NDM}$ and *bla*$_{CTX-M-15}$ genes, respectively. FIA and FIB are the plasmid replicon types. Black and white boxes indicate presence and absence of corresponding traits, respectively.
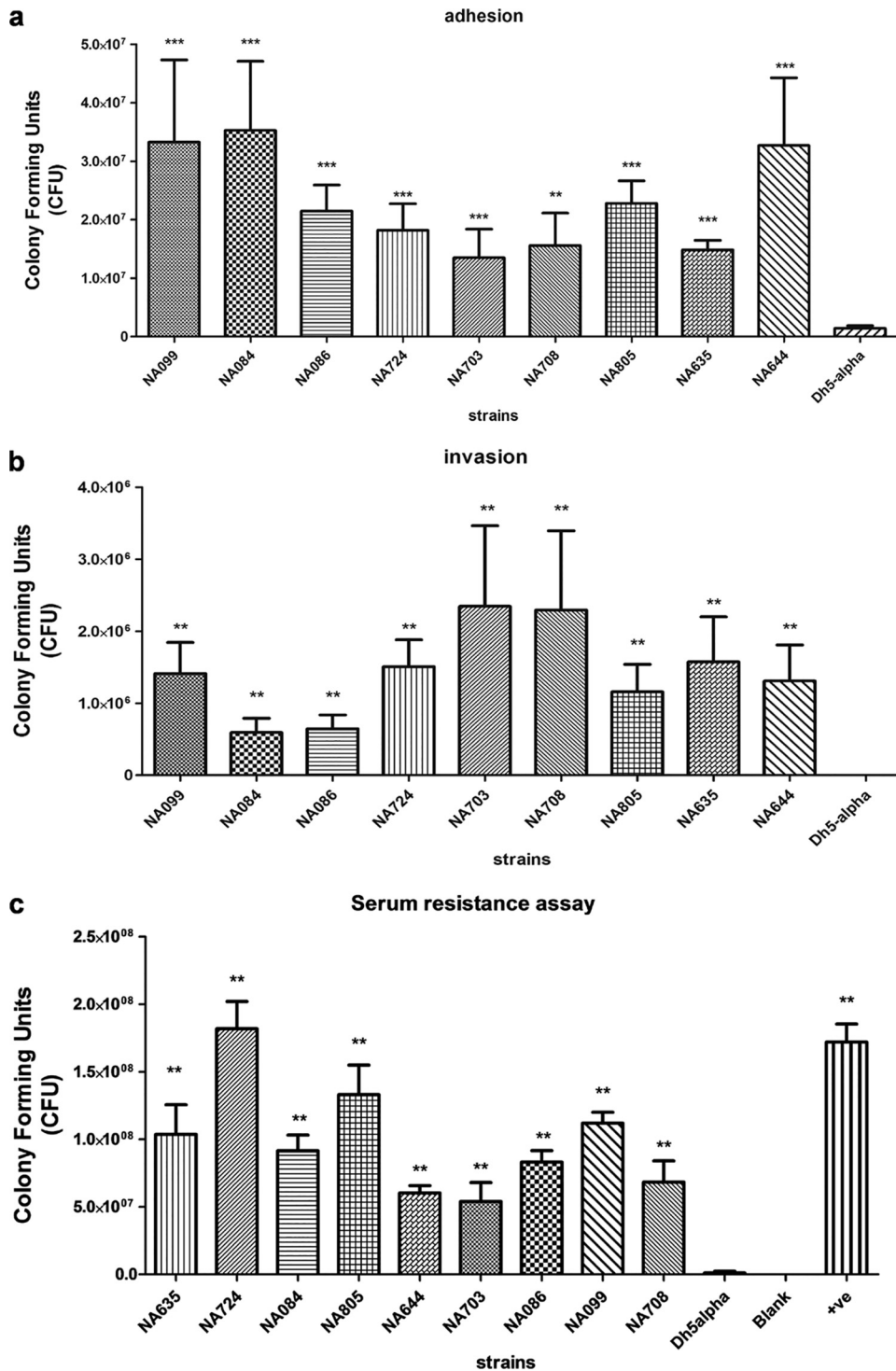
FIG 3 A subset of nine MBL-producing *E. coli* isolates was tested for three virulence-associated phenotypes: adhesion to human bladder epithelial cells (T24) at an MOI of 10 (a), invasion of human bladder epithelial cells (T24) (b), and resistance to 50% human serum for a 3-h time period (c). Adhesion and invasion assays were repeated three times in triplicates while the serum resistance assay was repeated twice in triplicates. All nine MBL-producing *E. coli* isolates demonstrated ExPEC-associated phenotypes. **, $P < 0.01$; ***, $P < 0.001$, for results compared to those with the control (DH5α).

teristics are represented in Table 2. The three NDM-1 strains belonged to ST101 while the two NDM-5 strains were characterized as ST167 and ST405. The whole-genome-based resistome reflected the high prevalence of resistance genes of all categories within all the strains, similar to the phenotypic drug resistance profile (Fig. 4). The virulome of these strains reflected moderate virulence wherein all the strains inherited genes belonging to adhesins, autotransporters, type three secretion systems

TABLE 2 The genome statistics and sequence types of five whole-genome-sequenced NDM-harboring strains comprising three NDM-1 and two NDM-5 strains

Strain	No. of raw reads	Genome coverage from filtered reads (×)	No. of contigs	ST	Genome size (no. of bases)	G+C content (%)	No. of CDS[a]	% coding capacity	No. of rRNAs	NDM type	Specimen source
NA084	1,741,426	73.25	109	101	5,147,001	50.59	5,000	86.8	13	NDM-1	Urine
NA086	1,643,674	63.13	115	101	5,147,281	50.59	4,994	86.9	13	NDM-1	Urine
NA099	2,406,806	104.68	155	101	5,197,495	50.57	5,063	85.8	17	NDM-1	Urine
NA703	2,401,628	92.94	211	167	5,263,381	50.66	5,186	86.0	16	NDM-5	Pus
NA724	2,784,910	118.36	166	405	5,424,442	50.55	5,273	87.0	9	NDM-5	Pus

[a] CDS, coding sequences.

(TTSS), and type 6 secretion systems (T6SS). NA099 harbors the least number of virulence genes while NA724 carries the maximum number of genes. The details are shown in Fig. 4. All the strains showed different phage contents and variable IS elements.

The upstream sequence analysis of these five NDM strains showed insertion elements such as IS*Aba125* upstream of NDM-1 and IS5 and truncated IS*Aba125* upstream of NDM-5 sequences.

NDM strains reveal high genomic diversity. PCR-based
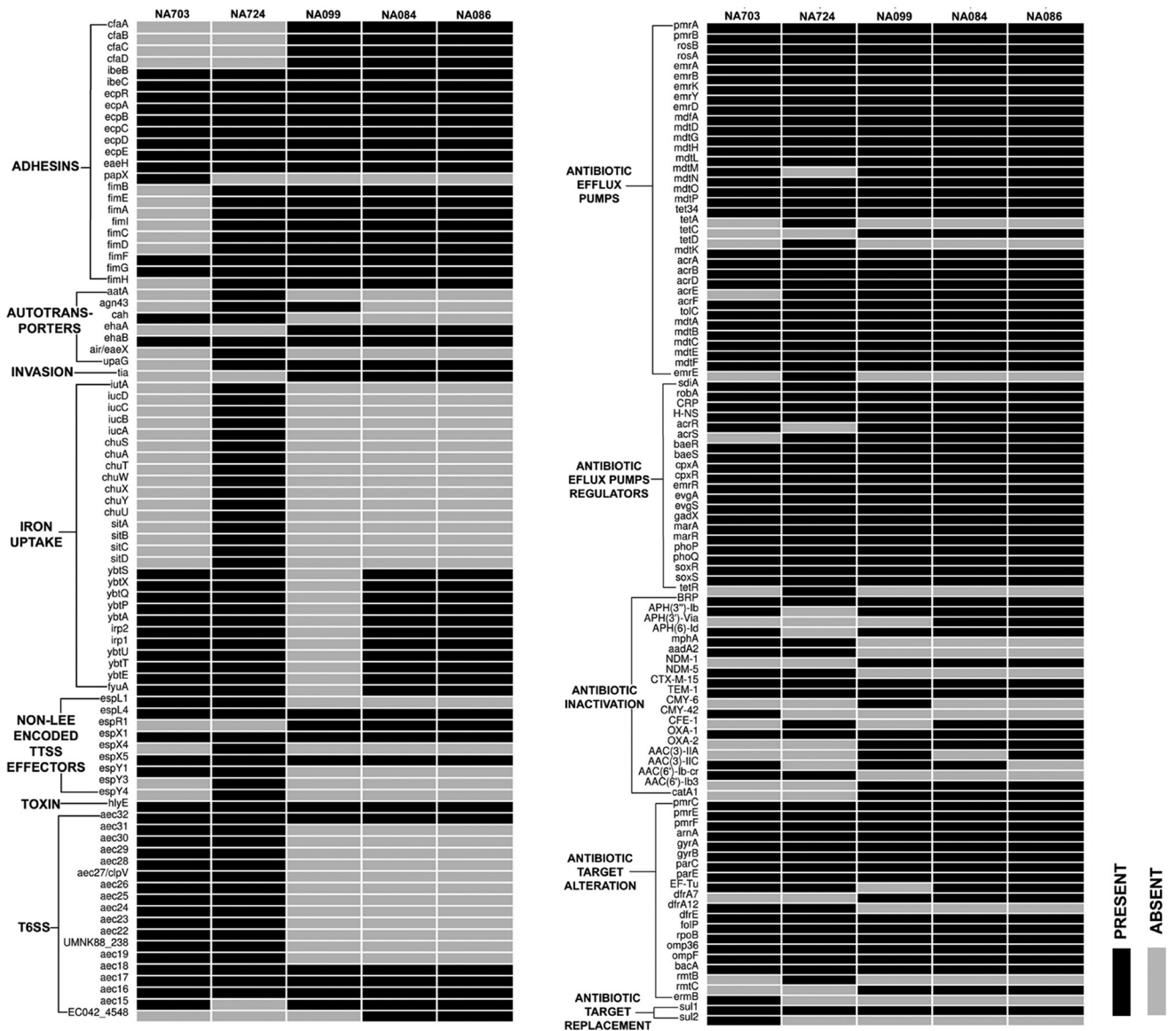

FIG 4 Whole-genome-based identification of virulence (left) and resistance (right) determinants of five MBL-producing *E. coli* isolates.
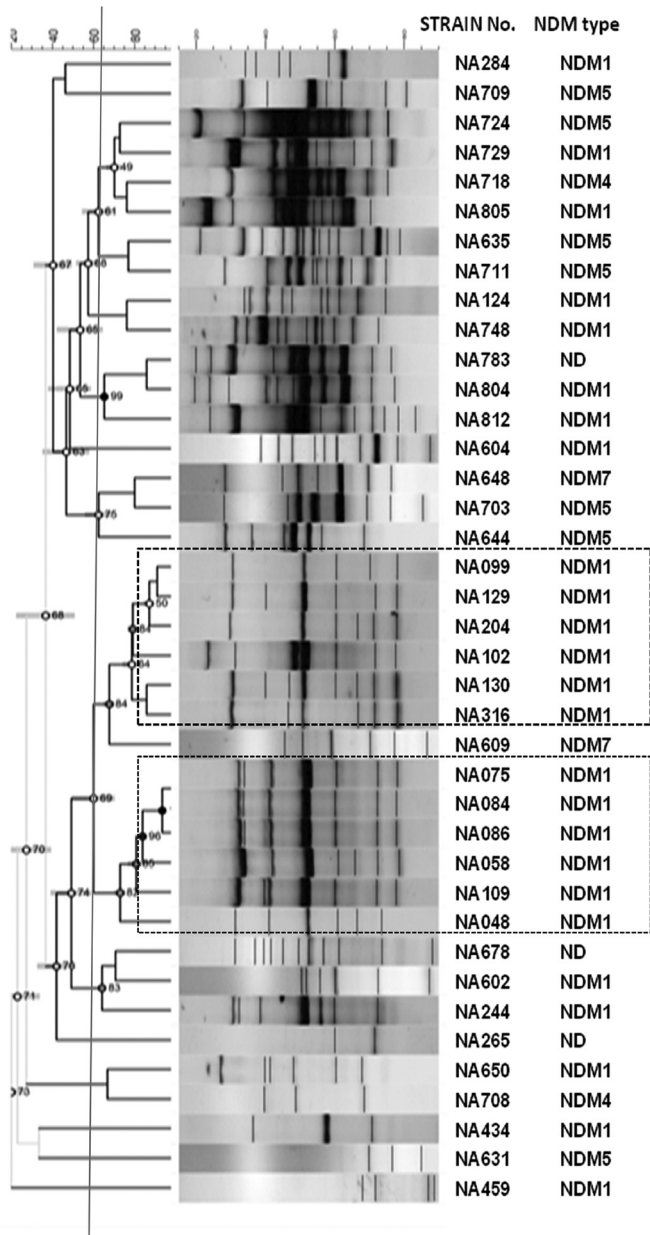
FIG 5 Dendrogram based on the phylogenetic analysis of 39 MBL-producing carbapenem-resistant clinical *E. coli* strains using ERIC-PCR banding analysis by BioNumerics, version 7.5 (Applied Maths, Belgium). Dotted boxes indicate clusters into which the majority of NDM-1 profiles segregated. ND, not determined.

idea that plasmids are the important means for NDM dissemination.

DISCUSSION

The emerging NDM, an acquired class B carbapenemase from among *Enterobacteriaceae*, has become a major public health concern worldwide also due to its medical and economic impact (49), and infections with NDM-positive pathogens pose a major health care problem, particularly for high-burden countries such as India. Previous studies from our group have shown an endemic *E. coli* infection burden and the presence of clonal group ST131 isolates with NDM-1 (23–25, 50). This study was aimed at detection, comprehensive characterization, and whole-genome sequence analysis of MBL-producing MDR organisms that can lead to successful infection. This study would likely be useful in guiding the formulation of control strategies involving antimicrobial stewardship and public health interventions in order to control the escalation of life-threatening MDR bacterial infections involving ExPEC organisms.

In general, production of MBL in *Enterobacteriaceae* shows an increasing prevalence pattern. However, the prevalence of carbapenem resistance among 510 *E. coli* isolates analyzed from 2009 to 2014 was found to be 7.6%, which was within the range of previously reported carbapenem resistance from India (51, 52); however, this prevalence might be variable in different geographical locations and different hospital settings. The dissemination of NDM carbapenemases involves several bla_{NDM} gene variants that are associated with different plasmid types among several Gram-negative species. A total of 14 NDM types have been reported globally, which were due to one or more point mutations (http://www.lahey.org/studies). In our study, we identified four NDM variants responsible for MBL production, with NDM-1 being the dominant variant, while other variants comprising NDM-4, NDM-5, and NDM-7 were observed with various frequencies. Previous reports from India have shown the presence of the NDM-6 type in addition to the above-mentioned NDM types (52). These four variants did not demonstrate any significant difference in MIC values for meropenem, nor did they show any specific association with different antimicrobial resistances. The carbapenems are considered the antibiotics of last resort for treatment of MDR bacterial infections; hence, bacteria which are resistant to these agents are often termed superbugs as they possess a resistance phenotype against different classes of broad-spectrum antibiotics. The antibiogram of these 39 isolates (Fig. 1) reflected a similar scenario wherein all MBL-producing strains (100%) revealed pan-drug resistance phenotypes as they were completely resistant to almost all the empirically used antibiotics. Nevertheless, all the strains were susceptible to colistin, and a majority of them were susceptible to fosfomycin. Polymyxins are the antibiotics of last resort to treat infections caused by MBL-producing *Enterobacteriaceae* (53), and as combination therapies are being advocated for treatment of MDR pathogens (54), the dual combination of colistin and fosfomycin could be the best option for effective treatment of carbapenem-resistant infections, which also minimizes the chances of resistance development.

The spread of antibiotic resistance genes is mediated by horizontal gene transfer (HGT) between bacteria (55). As multidrug-resistant plasmids serve as the most efficient means for dissemination of resistance traits via HGT, we also observed that the NDM genes and other resistances were transferred (via plasmids)

genotyping was used to analyze the genetic relationships of the organisms. The ERIC-PCR-based dendrogram of these strains (Fig. 5) revealed high diversity among them. However, when strains were grouped at a lower threshold of ~65% identity, two diffusely clonal clades were observed. As expected, all the strains in this cluster harbored NDM-1 genes. Further, the *in silico* multilocus sequence typing (MLST) analysis of whole-genome sequences identified all three strains to be ST101. Overall, this cluster might include clonally related ST101 strains which are strongly associated with the NDM genotype. No such clusters were obtained for any other NDM variants. The above observations also support the

to recipient strains, as confirmed genotypically and phenotypically by our conjugation experiments (Fig. 2). ERIC-PCR-based clusters revealed the presence of NDM genes in *E. coli* isolates of mostly unique genotypes, suggesting that they are clonally unrelated and thereby have similar propensities to acquire the NDM genes (Fig. 5). Recent reports have shown a greater association of the NDM-1 genotype with sequence type 101, which is consistent with our findings as we also observed three NDM-1-bearing *E. coli* isolates belonging to ST101 (56, 57). We identified NDM-5 to be present in *E. coli* isolates belonging to ST405 and ST167. These sequence types were also linked to NDM dissemination worldwide (58–60). Further, we found that the NDM-1 sequences were preceded by the IS*Aba125* insertion element which belongs to the IS*30* family while NDM-5 has an IS*5* insertion element upstream of its sequence, followed by sequences of IS*Aba125*. IS*Aba125* is known to be located upstream of NDM in both *Acinetobacter* and *E. coli*, similar to our observations. It has also been reported that the IS*5* element is followed by a truncated IS*Aba125* sequence upstream of the NDM gene (61, 62).

The virulence phenotypes such as adhesion to and invasion of epithelial cells play an important role in initiation and establishment of infection. The virulence gene complement as detected at the whole-genome level (Fig. 4) and the adhesion/invasion (Fig. 3a and b) capabilities of the strains toward human urinary bladder epithelial cells demonstrate their pathogenic potentials and associated risks during infection as these strains are resistant to all empirically used antibiotics. Peirano et al. have also shown the ability of CTX-M- and NDM-positive strains to adhere to Caco2 and HepG2 cell lines (63). The ability to resist the bactericidal effect of serum complement as determined by serum resistance assays (Fig. 3c) points toward the enhanced survival advantages of these strains at different extraintestinal infection sites.

In summary, our findings demonstrated that the human extraintestinal pathogenic MBL-producing *E. coli* strains were associated with promiscuous plasmid types, resulting in a diverse range of clones harboring different variants of the NDM gene. Furthermore, whole-genome sequencing and genetic fingerprinting suggest that bla_{NDM-1} may also disseminate via dominant clones such as ST101. The genomes newly sequenced herein would be an important addition to the existing uropathogenic *E. coli* (UPEC)/ExPEC genome archives and would be helpful in future studies involving comparative genomics and molecular epidemiology. Given that the emergence of newer variants of NDM-1 in countries of endemicity and their association with different ExPEC infections represent an alarming problem, large-scale surveillance and molecular genotyping studies are needed in order to develop strategies to circumvent the emergence of dominant clones potentially leading to institutional outbreaks and nosocomial epidemics of the future.

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