Whole genome-based genetic insights of *bla*$_{NDM}$ producing clinical *E. coli* isolates in hospitals settings of Pakistan

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Abstract

Carbapenem resistance among Enterobacterales has become a global health concern. Clinical *Escherichia coli* isolates producing the metallo β-lactamase NDM have been isolated from two hospitals in Faisalabad, Pakistan. These *E. coli* strains were characterized by MALDI-TOF, PCR, antimicrobial susceptibility testing, XbaI and S1 nuclease pulsed-field gel electrophoresis, conjugation assay, DNA hybridization, whole genome sequencing, bioinformatic analysis and *Galleria mellonella* experiments. Thirty-four *bla*$_{NDM}$ producing *E. coli* strains were identified among 52 nonduplicate carbapenem-resistant strains. More than 90% of the isolates were found to be multidrug resistant by antimicrobial susceptibility testing. S1 PFGE confirmed the presence of *bla*$_{NDM}$ gene on plasmids ranging from 40kbps to 250kbps, and conjugation assays demonstrated transfer frequencies of *bla*$_{NDM}$ harboring plasmids ranging from $1.59x10^{-1}$ and $6.46x10^{-8}$ per donor. Whole genome sequencing analysis revealed *bla*$_{NDM-5}$ as the prominent NDM subtype with the highest prevalence of *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *aadA2*, *aac(6')-Ib-cr*, and *tet(A)* associated resistant determinants. *E. coli* sequence types: ST405, ST361, and ST167 were prominent, and plasmid Inc types: FII, FIA, FIB, FIC, X3, R, and Y, were observed among all isolates. The genetic environment of *bla*$_{NDM}$ region on IncF plasmids included partial IS*Aba125*, the bleomycin *ble* gene, and a class I integron. The virulence genes *terC, traT, gad, fyuA, irp2, capU* and *sitA* were frequently observed and *G. mellonella* experiments showed that virulence correlated with the number of virulence determinants. A strong infection control management in the hospital is necessary to check emergence of carbapenem resistance in Gram negative bacteria.

Importance

We describe a detailed analysis of highly resistant clinical *E. coli* isolates from two tertiary care centres in Pakistan including carbapenem resistance as well as common co-resistance

mechanisms. South Asia has a huge problem with highly resistant *E. coli.* However, we find that though these isolates are very difficult to treat they are of low virulence. Thus the western world has an increasing problem with virulent *E. coli* that are mostly of low antibiotic resistance. Whereas, South Asia has an increasing problem with highly resistant *E. coli* that are of low virulence potential. These observations allow us to start to devise methodologies to limit both virulence and resistance and combat problems in developing nations as well as the western world.

Introduction

Antimicrobial resistance (AMR), particularly in Gram-negative bacteria, is one of the greatest global challenges to public health systems (1). Morbidity and mortality caused by multidrug-resistant (MDR) bacteria are increasing globally, with a recent study estimating the global burden of AMR at 4.95 million deaths in 2019 (2). Carbapenems have been recognized as last-resort antibiotics due to their broad-spectrum of antibacterial activity for severe infectious diseases caused by multidrug-resistant bacteria (3). However, their increased clinical use leads to the development of carbapenem-resistant Enterobacterales (CRE), responsible for healthcare-associated infections. CRE do not respond to commonly available antibiotics and are frequently associated with high mortality (4). In Asia, the resistance rate of Enterobacterales to imipenem and meropenem rose from 0.8%-1.2% and 1.0%-1.3%, respectively from 2001 to 2012 (5). In Pakistan carbapenem resistance of *E. coli* and *K. pneumoniae* had risen from 1%-5% and 3%-18% respectively from 2009 to 2014 (6). Different carbapenemases produce by these bacteria that inactivate carbapenems, NDM is the main carbapenemase found throughout South Asia and is the most clinically significant because of its rapid and ongoing evolution and global dissemination (7).

Since a *K. pneumoniae* NDM-bearing strain reported first time from a Swedish patient who had received prior treatment in New Delhi, NDM has spread worldwide with common links to South Asia (8), –48 variants of NDM (NDM-1–48) have been detected so far (9). NDM-1, 4, 5, 6, and 7 are most prevalent worldwide and NDM-5 is the most prevalent in South Asia and China (10). In contrast, several other types of carbapenemases, such as KPC, OXA-48, IMP, VIM, have found to be more common in other countries (11, 12). The highest distribution of NDM-positive species is observed in *K. pneumoniae* and *E. coli* (13). Notably, one of the primary reasons for the rapid emergence and spread of NDM is its close association with *E. coli* carried by the vast majority of humans, in addition to its close association with different mobile genetic elements such as insertion sequences, ISCR elements, plasmids, other transposons, and integrons. Conjugative plasmids such as Incompatibility groups (Inc) F, L/M, N, A/C, and X are commonly associated with the spread of *bla*$_{NDM}$ via horizontal gene transfer (HGT) (14).

The Indian subcontinent is the region that is of special concern for the presence and dissemination of *bla*$_{NDM}$ genes The Indian subcontinent is the most endemic region for the presence and spread of NDM-type MBLs, and prevalence rates of Enterobacterales producing *bla*$_{NDM}$ were found in a range of >30% in hospitals of India and Pakistan (15, 16). In Pakistan, a study in Karachi, reported that bacteria producing *bla*$_{NDM}$ were found to be responsible for 66%, and 57% mortality in neonatal and adult patients, respectively (17). Similarly, in Pakistan, another study reported the death of four out of nine neonates due to bacteria producing *bla*$_{NDM}$ genes (18). Several other studies have been carried out to examine the dissemination of *bla*$_{NDM}$ (19-22) in Pakistan and reported an increase in the prevalence of these genes. In 2020, a meta-analysis reported a 28% pooled proportion of clinical carbapenem-resistant Gram-negative bacteria from Pakistan (23).

In this study, we performed whole genome sequencing of clinical *E. coli* isolates producing *bla*$_{NDM}$ genes and reported the detailed genetic context of *bla*$_{NDM}$-carrying plasmids. This knowledge provides insight into genetic characteristics and potential transmissions of the plasmids among clinical *E. coli* isolates in Pakistan.

Material and methods

Ethical statement

This study was approved by the Institutional Biosafety Committee (IBC) D. No. 8025/ORIC of University of Agriculture, Faisalabad.

Isolation of carbapenem-resistant *E. coli* isolates

A total of 240 *E. coli* strains recovered from urine or pus cultures were collected from laboratories of two tertiary care hospitals in Faisalabad in 2019 and 2020 (39). These isolates were sub-cultured on CHROMagar media plates supplemented with 1µg/ml meropenem and incubated overnight at 37°C for purity checks, and isolation of carbapenem-resistant *E. coli* isolates.

Identification of bacteria

Matrix-assisted laser desorption ionization-TOF (MALDI-TOF) (Bruker Daltonics, Germany) was carried out for protein-based confirmation of bacteria at the species level.

Molecular identification of the *bla*$_{NDM}$ gene

DNA of *E. coli* isolates was subjected to PCR to screen the presence of *bla*$_{NDM}$ gene using primers and conditions as described previously (24).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of all carbapenem resistant *E. coli* isolates was performed by disk diffusion method using the Muller Hinton agar plates against 12 antimicrobials. Broth

109 microdilution, and E-tests, were performed to analyze the susceptibility of isolates against colistin
110 and tigecycline respectively. *E. coli* strain ATCC 25922 was used as the control strain. Breakpoints
111 of all antibiotics were interpreted according to EUCAST criteria (25).

112 **Pulsed-field gel electrophoresis (PFGE) analysis**

113 All carbapenem resistant *E. coli* isolates were further subjected to XbaI and S1 PFGE analysis as
114 described by CDC 2016 (www.cdc.gov/pulsenet/pdf /ecoli-shigella-salmonella-pfge-protocol-
115 508c.pdf). Briefly, agarose blocks of bacterial DNA was prepared in 1% SeaKem Gold Agarose
116 (Lonza, Rockland, ME, USA) with 0.5X TBE (Tris–borate–ethylene) buffer, digested with XbaI
117 and S1 nuclease enzymes and separated by electrophoresis using a CHEF Mapper XA Apparatus
118 (Bio-Rad, Hercules, CA, USA) at 6 V cm^{-1} at 14 °C, with an initial pulse time of 4 s and a final
119 pulse time of 45 s for 22 h. In-gel DNA–DNA hybridization with *bla*$_{NDM}$ probe labeled with ^{32}P
120 was carried out to determine the genetic location of carbapenem-resistant genes as described
121 previously (26).

122 **Conjugation experiment**

123 Conjugation experiments were carried out to determine the transferability of plasmid-mediated
124 *bla*$_{NDM}$ genes using sodium azide-resistant *E. coli* J53 as the recipient strain, as previously
125 described (27). Briefly, isolates were grown on chromogenic media plates with 1µg/ml meropenem
126 (AstraZeneca, London, UK) and *E. coli* J53 on chromogenic media with 200µg/ml sodium azide.
127 Pure cultures were propagated by picking a single colony and inoculating in 10ml of LB broth for
128 incubation at 37°C for 18hrs, with shaking at 200rpm. Mating was undertaken by mixing 1.5 ml
129 of overnight culture of the resistant strain with 1ml of *E. coli* J53 bacterial culture and 2ml of LB
130 broth. After incubation for 18hrs at 37°C, 10µl was used to inoculate plates containing 200µg/ml
131 sodium azide and 1µg/ml meropenem to select transconjugants. Single colonies were subcultured

and analysed for the presence of resistance genes by PCR. Transfer frequencies were calculated by the colony forming unit (CFU) count of transconjugants against the CFU count of the donor.

Whole-genome sequencing and bioinformatics analysis

Total gDNA was extracted from an overnight culture (2 ml) on a QIAcube automated system (Qiagen). Following extraction, gDNA was quantified by fluorometric methods using a Qubit (ThermoFisher Scientific, USA), with quality ratios of gDNA (A260/280 and 260/230) determined via Nanodrop (ThermoFisher Scientific, USA). Genomic DNA libraries are prepared for whole-genome sequencing using the NexteraXT kit (Illumina), as described by the manufacturer. Paired-end sequencing was performed using the Illumina MiSeq platform (MiSeq Reagent V3 Kit; 2 × 300 cycles). For each *E. coli* isolate, at least 20× coverage was generated. Raw sequence reads were trimmed using Trim Galore and the genomes were de novo-assembled into contigs using SPAdes (3.9.0) with a pre-defined kmers set (28). Raw reads were also assembled with Geneious (10.0.9; Biomatters Ltd.) de novo assembler, set at medium sensitivity for analysis of paired Illumina reads. Geneious was used to map both sets of contigs to reference genes identified by closest BLAST homology and to annotate genes from closest homologs in NCBI Genome database. Resistance genes were identified using Resfinder within CGE, and plasmids were identified within the genome assembly and typed using Plasmidfinder (29).

To elucidate the genetic environments of *bla*$_{\text{NDM}}$ genes, 10 representative *bla*$_{\text{NDM}}$ isolates carrying plasmids were selected based on the phylogenetic analysis to perform MinION (Oxford Nanopore Technologies Ltd., Oxford Science Park, UK) sequencing to obtain the complete plasmid sequences. Large-scale bacterial gDNA was extracted. Two loops of a full bacterial colony in 9.5 mL of TE buffer were mixed with 50 µL proteinase K (20mg/mL) and 0.5 mL of 10% SDS. After incubation at 37°C for 1 hour, 1.8 mL heated 5M NaCl was added and was incubated at 65°C for

5 minutes. The suspension was treated with 1.5 mL of heated CTAB/NaCl and was incubated at 65°C for 20 minutes. Then an equal volume of chloroform was added to the mixture followed by gently shaking for 1 hour. The mixture was centrifuged at 13,000 rpm for 15 minutes. The supernatant was taken, and equal volume of isopropanol was added until the clumping of gDNA was visualized. DNA was washed with 70% ethanol, and 200 µL of water was added to dissolve the gDNA. DNA library was prepared by pooling all barcoded samples to aim for a final DNA concentration >500ng/µL and 1 µL of RAD was added to DNA. A final mixture of 75 µL (34 µL sequencing buffer, 30 µL water and 11 µL DNA library) was loaded into the flow cell. MinION device was connected to MinKNOW GUI to obtain the reads. The raw data in fast5 format were base called with the high-accuracy mode and demultiplexed using Guppy 4.2.2 (30). Unicycler (0.4.4) was used to yield hybrid assembly using both Illumina short reads and minION long reads (31). This process included assembling the long reads with Flye v2.8 and following a 5-round polishing using Pilon (50) with the Illumina short reads of the same sample (32). Comparisons of the complete *E. coli* plasmids were visualized using BRIG and EasyFig (33).

***Galleria mellonella* pathogenicity model**

Pathogenicity of carbapenem-resistant *E. coli* isolates belonging to ST-405, ST361, and ST-167 was examined in a Galleria model. Larvae of the wax moth *Galleria mellonella* were used as an animal model for disease resulting from infection challenges of the test strains as described previously. Briefly, strains were standardised in suspensions equating to $1x10^7$, 10^6, and 10^5 CFU/ml. Using a Hamilton Syringe, 10µl of each suspension were injected into the hemocoel of the *G. mellonella* larvae, and larvae were incubated in the dark at 37ºC for 72 hours, and the amount of died and alive worms was checked every 24 hours. Death was denoted when larvae no longer responded to touch. In addition to this, 3 more groups of 10 larvae were injected with non-

pathogenic *E. coli* ATCC25922 to evaluate whether larvae were killed by non-infection related reactions, with 10 µl of PBS to measure any lethal effects due to physical injury, and positive control inoculation with pathogenic strain KP-2 belonging to ST-131. Results were analysed by Kaplan-Meier survival curves (GraphPad Prism statistics software) (34).

Data availability

The genomes of *E. coli* isolates have been submitted to NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under the bioproject PRJNA932156.

Results

General characteristics, molecular identification, and antibiotic susceptibility of CR-EC isolates

Fifty-two non-repetitive carbapenem resistant *E. coli* isolates were isolated from two hospitals over one year. These non-duplicated isolates were mainly isolated from urine (79%) followed by pus (21%) cultures. Similarly, the percentage of CR-EC isolated from female and male samples was found to be 73% and 27% respectively. The age of patients ranged from 14-68 years, with a mean of 49 years. The highest number of isolates was found to be in the 40-49 age range (32%) while the lowest number of isolates was found in the 20-30 age range (6%).

Among the 52 carbapenem resistant *E. coli* isolates, 34 strains were demonstrated to be bla_{NDM} producers via specific bla_{NDM} PCR. Antimicrobial susceptibility testing revealed that all 34 *E. coli* isolates were MDR strains, and they were resistant to multiple categories of antibiotics (n>3) (Table S1 in supplemental material). Therefore, each isolate carried at least three categories of resistance genes associated with resistance phenotype (Table 1). Almost all these isolates were non-susceptible to fluoroquinolones (95-100%), cephalosporins (87-95%) and penicillins (82-

97%). Moreover, over half of the isolates were resistant to aminoglycosides (57%). The MICs results of CR-EC isolates revealed 100% susceptibility to tigecycline and colistin.

Pulsed field gel electrophoresis

XbaI PFGE analysis of carbapenem-resistant isolates (n=34) categorized them into 5 different clusters. Cluster A (PK-5027, PK-5034, PK-5068, and PK-5172) includes 4 isolates. Cluster B (PK-5092, PK-5171, and PK-5202) includes 3 isolates. Cluster C (PK-5093, and PK-5095), D (PK-5140, and PK-5141) and E (PK-5112, and PK-5198) include 2 isolates each. All other 21 isolates appeared to be singletons. S1-PFGE analysis showed that *bla*$_{NDM}$ was located on a plasmid with varying lengths. The number and sizes of plasmids in *E. coli* isolates were found to be between 1 to 5 and 40kbps to 250kbps, respectively (Table S2).

Conjugation

The conjugation experiment confirmed that *bla*$_{NDM}$ was able to transfer successfully into *E. coli* J53 strain. The range of conjugation frequencies for all isolates was observed between $1.59x10^{-1}$ and $6.46x10^{-8}$ per donor (Table S2).

Resistance determinants

Analysis of antibiotic resistance genes revealed the presence of *bla*$_{NDM-5}$ in 91%, (n=31/34) while the percentage prevalence of *bla*$_{NDM-1}$, *bla*$_{NDM-20}$, and *bla*$_{NDM-21}$ was 3% (n=1/34) each. Between 4-20 antibiotic resistance genes were found in each isolate. PK-5171 was found to harbor 20 antibiotic-resistance genes belonging to 9 different classes of antibiotics, followed by two isolates PK-5127 and PK-5136, and four isolates PK-5081, PK-5112, PK-5138, and PK-5198 were found to harbor 17 and 16 genes respectively. The 34 sequenced isolates harbored a plethora of resistant genes, including β-lactamases *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *bla*$_{EC-15}$, *bla*$_{TEM-1}$, *bla*$_{EC-8}$, *bla*$_{EC}$, *bla*$_{CMY-145}$, *bla*$_{EC-18}$, *bla*$_{CTX-M-139}$, *bla*$_{CTX-M-101}$, *bla*$_{OXA-181}$, *bla*$_{CMY-42}$, *bla*$_{CMY-102}$ and *bla*$_{CMY-131}$ *bla*$_{OXA-10}$ *bla*$_{CTX-}$

M-103, aminoglycosides (*aadA2* 65%, *aac(6')-Ib-cr*, *aadA5*, *aadA1*, *aph(3'')-Ib,* and *aph(6)-Id*, *aac(3)-Iid*, *aadA11, aadA16,* and *aac(3)-Iia*, fluoroquinolones (*qnrS1, qnrB6, qepA1, qepA6, qepA8,* and *qepA9*), sulfonamides (*sul1, sul2,* and *sul3*), trimethoprim (*dfrA12, dfrA14,* and *dfrA17*), phenicols (*catB3, catA1,* and *cmlA1*), tetracyclines (*tet(A), tet(B),* and *tet(34)*) and macrolides (*mph(A)*). Genes $bla_{\text{OXA-1}}$ (73%, n= 25/43) and $bla_{\text{CTX-M-15}}$ (50%, n= 17/34) were found to be the most predominant β-lactamases among the isolates. All isolates harbored more than one *bla* in different combinations. Aminoglycoside resistance was mostly mediated by *aadA2* (65%, n=22/34), and *aac(6')-Ib-cr* (47%, n=16/34) that confers resistance to both aminoglycosides and fluoroquinolones. Among the tertacycline resistant genes, Gene *tet(A)* (67%, n=23/34) was the most common tetracycline resistance gene carried by isolates (Table 1).

Virulence genes

A total of 17 various virulence genes were detected across 34 *E. coli* isolates in different combinations of 4 to 16 genes. The most frequent genes found among the isolates were *terC* (100%, n=34/34), *traT* (88%, n=30/34), *gad* (79%, n=27/34), *fyuA* and *irp2* (61%, n=21/34), *capU* (52%, n=18/34) and *sitA* (50%, n=17/34). Isolates detected with more than 10 virulence genes PK-5138 (n=16), PK-5136 (n=15), PK-5081 and PK-5127 (n=14), PK-5178 (n=13), and PK-5179 (n=12) belong to ST-405. Only two isolates PK-5171 and PK-5037, with virulence genes of 11 and 10 respectively, belong to ST-167 (Table 1).

Replicon typing

Screening of plasmid replicons among 34 *E. coli* isolates using the PlasmidFinder database detected 9 plasmid replicons, including FII, FIA, FIB, FIC, X3, R, Y, Col, and p0111. FIA was the predominant replicon identified in 80% (n=27/34) of isolates followed by Col, FII, FIC, and FIB replicon types, with 61% (n=21/34), 52% (n=18/34), 47% (n=16/34), and 38% (n=13/34)

respectively. There were 1 (n=1), 2 (n=3), 3 (n=10), 4 (n=14), and 5 (n=6) plasmids detected in the isolates (Table 1).

Phylogenetic analysis

WGS analysis provided comprehensive information for the 34 *bla*$_{NDM}$ carrying *E. coli* and their phylogenetic relationship. Phylogenetic relationships among *E. coli* isolates were determined by using the online tool CSI Phylogeny (1.4 version) (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). The pipeline comprises of various freely available programs. The paired-end reads from each isolate were aligned against the reference genome, using Burrows-Wheeler Aligner (BWA), SAMtools, "mpileup" command and bedtools. The single nucleotide polymorphism (SNP) based phylogenetic tree was generated by calling and filtering SNPs, site validation and phylogeny based on a concatenated alignment of the high-quality SNPs. For inferring phylogeny, the analysis was run with the standard parameters and the NCTC11129 strain genome (GenBank accession number NZ_LR134222.1) was used as a reference sequence. The analysis was run with the default parameters; a minimal depth at SNP positions was ten reads along with a relative depth at SNP positions of 10%, a minimal distance between SNPs was of 10 bp, a minimal SNPs quality was 30 and a minimal Z-score was of 1.96, a minimal SNP quality was 30 and a minimal read mapping quality was of 25. The Z-score expresses the confidence with which a base was called at a given position and the Z-score was 1.96. Number of SNPs exhibited among closely related isolates were calculated using distance matrix file generated as a result of phylogeny (35). The output core alignment file was used to construct the Maximum-likelihood tree with 1000 bootstrap replications, by using MEGA-X v 10.0.5 (https://www.megasoftware.net/home) (36). The phylogenetic tree of the alignments was visualized and edited by iTOL v 4.4.2 software (https://itol.embl.de) (37). Genomes of the sequenced isolates covered 70% of the NCTC11129

reference genome. The phylogenetic tree showed that among 34 distinct *E. coli* strains, 2 belong to unknown STs, and 32 belong to 8 ST types: ST405 (n=11), ST167 (n=7), ST361 (n=7), ST156 (n=2), ST2851 (n=2), ST1702 (n=2), and ST2450 (n=1) (Fig. 1). Isolates exhibiting the same sequence types are grouped closely in the resulting tree. Notably, the reference genome grouped with three isolates belonging to ST156 and unknown ST. Interestingly 2 isolates PK-5055 and PK-5037 from ST-1702 and 1 isolate of unknown ST grouped with isolates belonging to ST167. We noticed that 2 *E. coli* strains belonging to unknown STs were isolated from urine culture. The other 25 and 7 *E. coli* strains isolated from urine and pus cultures, respectively, were distributed among all 8 sequence types. We further determined the SNPs distance of the core genome. SNPs matrix in the final dataset revealed a minimum of 6 SNPs and a maximum of 41296 SNPs detected between all examined genomes. The core alignment showed that in ST405, isolates PK5140 and PK5141 differed from each other by 9 SNPs. In ST361, there was a difference of 26 SNPs between the isolates PK-5027 and PK-5034, and 5099 and 5172. Similarly, in ST167 isolates 5112 and PK-5198, and in ST2851, isolates PK5224 and PK5238 differed by 28 and 71 SNPs (Table S3).

Comparative analysis of plasmids

Genomic DNA of 10 isolates (PK-5055, PK-5081, PK-5099, PK-5112, PK-5144, PK-5160, PK-5171, PK-5172, PK-5209, and PK-5224) were selected according to phylogenetic analysis to be sequenced with the MinION long-read platform for comparative analysis of plasmids. Basic information of *bla*$_{NDM}$ harboring plasmids in these isolates was summarized in table 2. Isolates PK-5055, PK-5224 and PK-5209 harbored *bla*$_{NDM-5}$, *bla*$_{NDM-5}$, and *bla*$_{NDM-21}$, gene respectively, on IncFII-IncFIA plasmid. In isolates PK-5081 and PK-5171 *bla*$_{NDM-5}$ gene was located on IncFII-IncFIA-IncFIB plasmid. Similarly, PK-5160, PK-5172, PK-5099, and PK-5144 harbored *bla*$_{NDM-1}$, *bla*$_{NDM-5}$, *bla*$_{NDM-20}$ and *bla*$_{NDM-5}$ gene respectively on IncHI2, IncFII, IncFIA, and IncFIB

plasmids respectively. In PK-5112 the *bla*$_{NDM-5}$ gene was found on the ColKP3-IncFII-IncFIA plasmid (Table 2). BLASTn analysis revealed that pPK-5099, pPK-5209, pPK-5224, pPK-5112, pPK-5055, and pPK-5172 shared homology with plasmid p52148-NDM-5 (Accession no. CP050384.1) of *E. coli* strain 52148 isolated from urine sample of human in 2019 in Prague with identity 99.70%, 99.23%, 99.63%, 99.91%, 99.90%, and 98.58% at coverage 100%, 96%, 91%, 80%, 74%, and 62% respectively (Figure 2A). BLAST search of pPK-5081 and pPK-5144 exhibited identity 99.94% and 99.89% at coverage of 98% and 87% with plasmid p_dm655_NDM5 (Accession no. CP095638.1) of *E. coli* strain dm655 isolated from human blood sample in Bangladesh in 2017 and plasmid pNDM_P30_L1_05.20 (Accession no. CP085061.1) of *E. coli* strain P30_L1_05.20 isolated from human rectal sample in UK in 2020 respectively (Figure 2B). Similarly, BLASTn analysis of pPK-5171 revealed its 99.93% identity with p675SK2_B (Accession No. CP027703.1), and pMB5823_1 plasmid (Accession No. CP103646.1) at 98% and 85% of coverage respectively (Figure 2C). Plasmids p675SK2_B and pMB5823_1 of *E. coli* strains 675SK2 and 961 were isolated from wastewater in Switzerland in 2016 and blood samples of humans in the USA in 2018, respectively. IncHI2 type *bla*$_{NDM-1}$ bearing plasmid pPK-5160-*bla*$_{NDM-1}$ was detected in PK-5160 strain. BLASTn analysis of pPK-5160 against the NCBI nr database showed that pPK-5160-*bla*$_{NDM-1}$ shared 99.71% identity at 86% and 85% coverage with plasmids pXJW9B277-HI2-N (Accession No. CP068042.1), and pL1 (Accession No. CP071712.1) of *E. coli* strains XJW9B277, and EC20017429 isolated from bovine cell culture in China in 2018 and gastroenteritis sample of human in Canada in 2017 respectively (Figure 2D).

Genetic environment of *bla*$_{NDM}$ gene

The genetic environment around the *bla*$_{NDM}$ gene located on IncF plasmids can be classified into three types of groups. These regions carrying by *bla*$_{NDM}$ gene were surrounded by IS*26*. For group

A (*IS26-ΔISAba125-bla*$_{NDM}$-*ble*$_{MBL}$-*trpF-ISCR1-sul1-qacE-aadA2-dfrA12-IntI1*-IS26-*ΔTnAS1*) the flanking genetic structure of *bla*$_{NDM}$ gene was composed of an *IS26* and incomplete *ISAba125* interrupted by insertion sequence ISCR1 and the genes *ble*$_{MBL}$ (bleomycin resistance), *trpF (*phosphoribosylanthranilate isomerase), *sul1 (*sulfonamide-resistant dihydropteroate synthase), *qacE (*quaternary ammonium compound resistance protein), *aadA2 (*aminoglycoside nucleotidyltransferase), *dfrA12 (*dihydrofolate reductase), and *IntI1* (class 1 integron integrase) located downstream. Furthermore, this group also has downstream addition of *ΔTnAS1* associated with *IS26.* Compared with group A, group B (*IS26-IS26-ΔISAba125-bla*$_{NDM}$-*ble*$_{MBL}$-*trpF-ISCR1-sul1-qacE-aadA2-dfrA12-IntI1*-IS26-*ΔTnAS1*) includes another *IS26* upstream addition and completed downstream deletion of *ΔTnAS1.* Group C (*IS26-ΔISAba125-bla*$_{NDM}$-*ble*$_{MBL}$-*trpF-ISCR1-sul1-qacE-aadA2-dfrA12-IntI1-ΔTnAS3-IS26*) includes the downstream addition of *ΔTnAS3* associated with oppositely directed *IS26* (Figure 3).

One IncHI2 type *bla*$_{NDM-1}$ bearing plasmid pPK-5160-*bla*$_{NDM-1}$ was also identified in PK-5160 strain. The core genetic environment of *bla*$_{NDM-1}$ gene in this plasmid contains multiple antibiotic resistance genes including *tetR*, *tetA*, *bla*$_{NDM-1}$, and *ble*$_{MBL}$ surrounded by IS26 and IS3000 upstream and downstream from *bla*$_{NDM-1}$ (Figure 4).

Clinical *E. coli* isolates and *Galleria mellonella* mortality

Pathogenicity of all clinical *E. coli* strains belonging to the ST-405 (n=11), ST-361 (n=8), ST-167 (n=7) was determined in *Galleria mellonella* larvae, dose titration was performed with culture 10^5 to10^7 colony forming units. Two groups of 10 larvae were also injected with PBS and KP-2, a pathogenic strain, as a negative and positive control respectively. Percentage survival of worms were observed for post-infection 72 hours. Dose dependent survival was observed, as inoculum concentration 10^7 killed more larvae than 10^6 and 10^5. PBS control injected larvae all remained

alive over the 3 days' time course. In contrast, KP-2 killed 80% of larvae during the time course. Larvae injected with isolates of ST-405, ST-167 and ST-351 showed more than 70%, 70%, and 80% survival, respectively (Figure 5).

Discussion

AMR has been referred to as *"the silent tsunami facing modern medicine"*. This study was designed to provide insights into the genomic epidemiology of clinical *E. coli* isolates in hospital settings in Pakistan by a whole-genome sequencing approach. *E. coli* is responsible for causing multiple community-acquired and nosocomial infections, including bacteremia, UTI, septicemia, wounds and catheter-associated infections (38). In the current study, the majority of *E. coli* isolates were isolated from samples of urine (79%) and pus (21%). These findings agree with another study in Pakistan that found the prevalence of ExPEC isolates of 62.7% and 24.3% from urine and pus samples, respectively (39). Another study performed in Pakistan showed that *E. coli* causes 73% of nosocomial UTIs (40). These outcomes differ with a study in Peshawar, Pakistan that documented the prevalence of *E. coli* isolates 12% and 21% from pus and urine cultures respectively whereas none of *E. coli* strains were isolated from blood cultures (41). These differences in prevalence of *E. coli* isolates from urine and pus cultures could be associated with variations in sample size, demographic characteristics, and methodology of research or perhaps virulence of locally carried strains.

The emergence of MDR *E. coli* isolates is causing therapeutic failures that is a serious public health threat and leads to high morbidities and mortalities in hospital settings (42). This study reported a high resistance rate for fluoroquinolones (95-100%), cephalosporins (88-95%), penicillins (82-97%), and aminoglycosides (58%). These findings are in agreement with the recent studies reporting increasing AMR in the South Asia region (43, 44). A recent study analyzed the AMR

rates for GLASS specified pathogen/antimicrobials combination from Pakistan (2006-2018) and reported a high resistance rate (>50%) to fluoroquinolones, 3rd generation cephalosporins amongst the *E. coli* and *K. pneumoniae* (45). The variation in results may be due to the variation of quality and standardization of antimicrobial sensitivity testing methods used in hospitals.

Our study exhibited the prevalence of multiple *bla*$_{NDM}$ variants such as *bla*$_{NDM-1}$, *bla*$_{NDM-5}$, *bla*$_{NDM-20}$, and *bla*$_{NDM-21}$, among clinical *E. coli* isolates. These findings are similar to other studies which indicate the widespread distribution of carbapenemases genes globally (46-49). In this study, NDM-5 was observed to be most prevalent among the carbapenem-resistant *E. coli* isolates, similar to other studies published in Pakistan and China (50,51). Since the isolation of *bla*$_{NDM-5}$ in Henan in 2013, its detection rate is continuously increasing, and now it has emerged as a prominent subtype of *bla*$_{NDM}$ (52). Several factors responsible for the dissemination of *bla*$_{NDM}$-carrying isolates include irrational use of broad-spectrum antimicrobials, self-medication, easy availability of antimicrobials at pharmacies without prescription of doctors, and substandard antimicrobials. All these factors are also considered a prominent source of the horizontal spread of superbugs among individuals (53).

To have better knowledge regarding the virulence potential of isolates, it is necessary to know the occurrence of virulence genes among these isolates. The most frequently occurring virulence genes among carbapenem-resistant *E. coli* isolates in this study are *terC, traT, gad, sitA, fyuA, irp2, CapU* and *iss*. These findings differ from the studies conducted in Iran and Egypt which reported the presence of *traT, fimH,iutA, csgA*, *hlyA,* and *crl* genes more frequently in carbapenem resistant ExPEC isolates and revealed that UPEC isolates were more pathogenic than others (54). The prevalence of *traT* gene contributes to the serum resistance of isolates as they become able to avoid complement systems which increases the risk of causing septic shock and the rate of mortality

(55). Pathogenicity of *E. coli* isolates was further examined by establishing the *Galleria mellonella* model experiment. We observed dose dependent response on larval survival, as survival of larvae decreases by increasing the inocula of *E. coli* strains. In this study most isolates with the exception of PK-5081, PK-5127, PK-5138 and PK-5178 showed negligible pathogenicity. This is likely due to the fact that many of the *E. coli* strains carried in Pakistan are of low virulence potential with only a few strains possessing additional virulence factors such as *iucC, iutA, sat, kpsE, kpsMII_K5, papA_F43* found in more pathogenic strains listed above. Another study used the *G. mellonella* model to examine the pathogenicity of ExPEC isolates presented a notable correlation between the virulence potential of isolates and virulence gene repertoire. The higher number of virulence genes in ExPEC isolates was responsible for rapid death of the larvae (56).

We found diverse *E. coli* STs, the most predominant was ST-405 followed by ST-167 and ST-361. Our findings are similar to another study conducted in Pakistan that reported ST405 in *E. coli* isolates (57). The most pathogenic isolate of NDM-producing *E. coli* belonging to ST405 is most commonly present in Asia and other regions of the world (58-61). ST167 NDM-producing *E. coli* strains are causing infections worldwide (62,63), which created great interest and attention. Notably, *E. coli* strains carrying the bla_{NDM} gene belonging to ST167 have been reported in companion animals (64,65), which suggests the transmission of ST167 *E. coli* harboring $bla_{\text{NDM-5}}$ gene between humans and animals. Notably, we did not find any *E. coli* strain positive for bla_{NDM} gene associated with ST131 in the current study.

The plasmid replicon typing analysis revealed different replicon types including IncF, IncI1, IncI2, IncX3, and IncY. IncF replicon type was most common in this study with sub-replicons such as IncFIA, IncFII, IncFIC, and IncFIB. In previous studies, different plasmid types harboring bla_{NDM} gene reported include IncFIA, IncFIB (66), IncHI1, IncFIIA, and IncN (67), IncX3 (68), IncB/O

(69), IncFIC, IncF, and IncK (70), and IncY, IncA/C, and IncI1 (71). Plasmids can acquire different resistance genes or transposons and are responsible for the spread of high levels of AMR (72). Out of 34 isolates, 10 harbored-IncI1 plasmid, which belongs to the narrow range of host plasmid type and was only observed in Enterobacterales. Several studies have suggested that IncI1 plasmids predominantly carry genes encoding for AMR, particularly for the ESBLs genes (73,74).

Conclusion

Carbapenem-resistance has been considered as one of the most significant menaces to global healthcare, and the prevalence of NDM variants in clinical *E. coli* isolates has further increased this threat. Therefore, early detection of the *bla*$_{\text{NDM}}$ possessing *E. coli* isolates with any decreased sensitivity to the carbapenems is crucial for choosing most appropriate antibiotic therapy and applying additional efficient infection control measures. The limited use of antibiotics, particularly carbapenems and cephalosporins, may help to prevent the emergence of such resistance- patterns. Furthermore, robust, and comprehensive infection control management in the hospital is required to avoid such infections.

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Figures legends

Figure 1 Phylogenetic tree of all 34 *bla*$_{NDM}$-positive *E. coli* isolates

Figure 2 (A, B, C, and D). Circular comparison of different IncF plasmids bearing *bla*$_{NDM}$ gene

Figure 3 Three (A–C) major types of *bla*$_{NDM}$-bearing genetic contexts among the *bla*$_{NDM}$-bearing IncF-type plasmids

Figure 4 Genetic elements surrounding the *bla*$_{NDM}$ gene on IncHI2 plasmid

Figure 5 Kaplan–Meier curves A, B, and C showing the percentage of *G. mellonella* survival for 72 h post-infection with carbapenem-resistant strains of *E. coli* belonging to ST-405, ST-167, and ST-361, respectively

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680 **Table 1. Genomic characteristics of clinical *E. coli* isolates harboring *bla*$_{NDM}$ gene**

Isolate ID	MLST	Inc types of plasmids	Resistance genes	Virulence genes
PK-5027	ST-361	IncFII, IncY, IncFIA, IncI1	*tet(A), sul1, bla*$_{EC}$, *bla*$_{OXA-1}$, *bla*$_{CMY-145}$, *aadA2, aadA1, bla*$_{NDM-5}$, *mph(A), dfrA12, qepA1, qepA8,*	*capU, gad, sitA, terC, traT*
PK-5034	ST-361	IncFIA, IncFII, IncY, IncI1	*tet(A), sul1, bla*$_{EC}$, *bla*$_{CMY-102}$, *bla*$_{OXA-1}$, *aadA2, aadA1, bla*$_{NDM-5}$, *mph(A), dfrA12, qepA8, qepA6*	*capU, gad, sitA, terC, traT*
PK-5037	ST-1702	IncFIA, IncI1, ColRNAI, IncFIC	*tet(34), sul1, bla*$_{EC-15}$, *bla*$_{CMY-42}$, *aadA2, bla*$_{NDM-5}$, *dfrA12*	*capU, gad, cia, cib, iss, fyuA, hra, iss,*

				terC, traT, irp2,
PK-5052	ST-405	IncFIB, IncFIA, IncFII, p0111	*tet(34), tet(A), sul1, bla*$_{EC-8}$*, bla*$_{CTX-M-15}$*, bla*$_{OXA-1}$*, catB3, aadA2, aac(6')-Ib-cr, bla*$_{NDM-5}$*, mph(A), dfrA12,*	*chuA, kpsE, kpsMII_K5, fyuA, terC, traT, irp2,*
PK-5055	ST-1702	ColRNAI, IncFIA, IncFIC	*tet(34), sul1, bla*$_{EC-15}$*, bla*$_{TEM-1}$*, aadA5, bla*$_{NDM-5}$*, dfrA12, dfrA17,*	*capU, irp2, fyuA, gad, iss, hra, terC, traT*
PK-5068	ST-361	IncFII	*tet(A), sul1, bla*$_{EC}$*, bla*$_{CMY-145}$*, catA1, catA1, aadA2, aadA1, bla*$_{NDM-5}$*, mph(A), dfrA12, qepA8*	*capU, gad, sitA, terC, traT*
PK-5081	ST-405	IncFIA, IncFIB, p0111, IncFIC, Col(MG828)	*tet(34), tet(A), sul1, sul2, bla*$_{EC-8}$*, bla*$_{CTX-M-15}$*, bla*$_{TEM-1}$*,bla*$_{OXA-1}$*, catB3, aadA5, aph(3'')-Ib, aph(6)-Id, aac(6')-Ib-cr, bla*$_{NDM-5}$*, mph(A), dfrA17*	*kpsE, kpsMII_K5, chuA, eilA, fyuA, sat, sitA, iucC, iutA, papA_F43, terC, traT, iha, irp2*
PK-5092	ST-167	IncFIC, IncFIA, IncFIB, Col156, Col(BS512)	*tet(34), tet(A), sul1, bla*$_{EC-15}$*, bla*$_{CTX-M-15}$*, bla*$_{OXA-1}$*, catB3,*	*capU, celb, gad, hra, iss, terC, traT*

			aadA2, aac(6')-Ib-cr, bla$_{NDM-5}$*, mph(A), dfrA12*	
PK-5093	ST-156	IncFIB, Col(MGD2), ColRNAI, IncFIC	*tet(B), tet(34), sul1, bla*$_{EC-18}$*, bla*$_{TEM-1}$*, aadA2, bla*$_{NDM-5}$*, dfrA12, qepA9*	*iss, lpfA, fyuA, gad, irp2, traT, papC, terC*
PK-5095	ST-156	IncFIB, Col(MGD2), ColRNAI, IncFIC	*tet(34), tet(B), sul1, bla*$_{EC-18}$*, bla*$_{TEM-1}$*, aadA2, bla*$_{NDM-5}$*, dfrA12, qepA9*	*iss, lpfA, papC, fyuA, gad, irp2, traT, terC*
PK-5096	Unknown	IncFIA, IncFII, ColRNAI, IncI1	*tet(34), tet(A), sul1, sul2, bla*$_{EC-15}$*, bla*$_{CMY-131}$*, aadA2, aph(3'')-Ib, aph(6)-Id, bla*$_{NDM-5}$*, mph(A), dfrA12*	*capU, iss, terC, traT, hra, irp2, fyuA, gad*
PK-5099	ST-361	IncFII_1, IncFIA_1, IncI1	*sul1, bla*$_{EC}$*, bla*$_{OXA-1}$*, catA1, aadA1, aadA2, bla*$_{NDM-20}$*, dfrA12, qepA1, qepA8*	*capU, gad, sitA, terC, traT*
PK-5112	ST-167	IncFIC, IncFIA, ColKP3, ColRNAI, Col(BS512)	*tet(34), tet(A), sul1, bla*$_{EC-15}$*, bla*$_{CTX-M-15}$*, bla*$_{OXA-181}$*, bla*$_{TEM-1}$*, bla*$_{OXA-1}$*, catB3, aadA2, aac(6')-Ib-cr, bla*$_{NDM-5}$*, mph(A), dfrA12, ere(A), qnrS1*	*capU, fyuA, gad, irp2, iss, terC, traT*

PK-5116	ST-167	IncFIA, IncFII	*tet(A), sul1, bla*$_{\text{OXA-1}}$*, bla*$_{\text{CTX-M-15}}$*, catB3, aac(6')-Ib-cr, aadA5, bla*$_{\text{NDM-5}}$*, mph(A), dfrA17,*	*capU, gad, hra, terC, traT, iss, hlyE*
PK-5127	ST-405	IncFIA, IncFIC, Col(MG828)	*tet(34), tet(A), sul1, sul2, bla*$_{\text{EC-8}}$*, bla*$_{\text{CTX-M-139}}$*, bla*$_{\text{CTX-M-101}}$*, bla*$_{\text{TEM-1}}$*, bla*$_{\text{OXA-1}}$*, catB3, aadA5, aph(6)-Id, aph(3'')-Ib, aac(6')-Ib-cr, bla*$_{\text{NDM-5}}$*, mph(A), dfrA17*	*afaD, chuA, fyuA, iha, irp2, iucC, iutA, kpsE, kpsMII_K5, papA_F43, sat, sitA, terC, traT*
PK-5136	ST-405	IncFIC, Col(MG828), IncFIA	*tet(34), tet(A), sul1, sul2, bla*$_{\text{EC-8}}$*, bla*$_{\text{CTX-M-101}}$*, bla*$_{\text{TEM-1}}$*, bla*$_{\text{CTX-M-103}}$*, bla*$_{\text{OXA-1}}$*, catB3, aadA5, aph(6)-Id, aph(3'')-Ib, aac(6')-Ib-cr, bla*$_{\text{NDM-5}}$*, mph(A), dfrA17*	*iha, irp2, iucC, afaD, chuA, papA_F43, sat, sitA, fyuA, gad, iutA, terC, traT, kpsE, kpsMII_K5*
PK-5138	ST-405	IncFIC, IncFIA, p0111, Col(MG828)	*tet(34), tet(A), sul1, sul2, bla*$_{\text{EC-8}}$*, bla*$_{\text{CTX-M-15}}$*, bla*$_{\text{OXA-1}}$*, bla*$_{\text{TEM-1}}$*, catB3, aadA5, aph(3'')-Ib,*	*fyuA, gad, iha, irp2, afaD, chuA, eilA, iucC,*

				aph(6)-Id, aac(6')-Ib-cr, bla$_{NDM-5}$*, mph(A), dfrA17*	*papA_F43, sat, sitA, terC iutA, kpsE, kpsMII_K5, traT*
PK-5140	ST-405	IncFIA, IncFII	IncFIB,	*tet(34), tet(B), sul1, bla*$_{EC-8}$*, bla*$_{TEM-1}$*, bla*$_{CTX-M-15}$*, bla*$_{OXA-1}$*, catB3, aadA2, aadA5, aac(6')-Ib-cr, aac(3)-Iid, bla*$_{NDM-5}$*, dfrA12, dfrA17*	*chuA, irp2, sitA, terC, fyuA, kpsE, kpsMII_K5, gad, hra,*
PK-5141	ST-405	IncFIA, IncFII	IncFIB,	*tet(34), tet(B), sul1, bla*$_{EC-8}$*, bla*$_{CTX-M-15}$*, bla*$_{OXA-1}$*, catB3, aadA2, aac(6')-Ib-cr, aac(3)-Iid, aadA5, bla*$_{NDM-5}$*, dfrA12, dfrA17*	*chuA, fyuA, hra, irp2, kpsMII_K5, sitA, terC*
PK-5144	ST-405	IncFIC, IncFIB	IncFIA,	*tet(34), tet(A), sul1, bla*$_{EC-8}$*, bla*$_{CTX-M-15}$*, bla*$_{OXA-1}$*, catB3, aadA2, aac(6')-Ib-cr, bla*$_{NDM-5}$*, mph(A), dfrA12*	*afaD, chuA, fyuA, irp2, kpsE, kpsMII_K5, terC, traT*
PK-5151	ST-2450	IncX3, IncFIB, p0111	ColRNAI,	*tet(34), sul2, bla*$_{EC-15}$*, aph(6)-Id, aph(3'')-Ib, aac(3)-Iia, bla*$_{NDM-5}$	*capU, fyuA, gad, irp2, iss, sitA, terC*

PK-5152	ST-405	IncFIA, IncFIB, IncFII	*tet(A), sul1, bla*$_{CTX-M-15}$*, bla*$_{OXA-1}$*, catB3, aac(6')-Ib-cr, bla*$_{NDM-5}$*, mph(A), dfrA12*	*kpsMII_K5, chuA, fyuA, gad, kpsE, terC, traT, irp2*
PK-5160	Unknown	Col156, IncFIB, IncFII, IncI1	*sul1, bla*$_{OXA-10}$*, cmlA1, bla*$_{NDM-1}$	*celb, gad, lpfA, terC*
PK-5171	ST-167	IncFIA, IncR, IncFIB, IncFIC, ColRNAI	*tet(34), tet(B), tet(A), sul1, bla*$_{EC-15}$*, bla*$_{CTX-M-139}$*, bla*$_{OXA-1}$*, catA1, catB3, aadA16, aac(6')-Ib-cr, aadA2, aadA5, bla*$_{NDM-5}$*, mph(A), dfrA27, dfrA12, dfrA17, qnrS1, qnrB6*	*capU, fyuA, gad, hra, irp2, iss, iucC, iutA, sitA, terC, traT*
PK-5172	ST-361	IncI1, IncFIA, IncFII	*tet(A), sul1, bla*$_{EC}$*, bla*$_{CMY-102}$*, bla*$_{OXA-1}$*, bla*$_{CMY-145}$*, catA1, aadA1, aadA2, bla*$_{NDM-5}$*, mph(A), dfrA12, qepA8*	*capU, gad, sitA, terC, traT*
PK-5176	ST-167	IncFIC, IncFIA, IncX3, Col(MG828)	*tet(34), tet(A), sul1, bla*$_{OXA-1}$*, bla*$_{EC-15}$*, bla*$_{CTX-M-15}$*, catB3, aac(6')-Ib-cr, aadA5, bla*$_{NDM-5}$*, mph(A), dfrA17*	*capU, gad, hra, iss, terC, traT*

PK-5178	ST-405	IncFIC, IncFIA, p0111, IncFIB, Col(MG828)	*tet(34), tet(A), sul1, sul2, bla*$_{EC-8}$*, bla*$_{CTX-M-15}$*, bla*$_{OXA-1}$*, bla*$_{TEM-1}$*, catB3, aadA5, aph(6)-Id, aph(3'')-Ib, aac(6')-Ib-cr, blaNDM-5, mph(A), dfrA17*	*chuA, fyuA, iha, irp2, iucC, iutA, kpsE, kpsMII_K5, papA_F43, sat, sitA, terC, traT*
PK-5179	ST-405	Col(BS512), IncFII	*tet(B), sul1, bla*$_{CTX-M-15}$*, aadA2, bla*$_{NDM-5}$*, dfrA12*	*chuA, fyuA, gad, eilA, irp2, kpsE, kpsMIII_K98, papA_F43, papC, sitA, terC, traT*
PK-5196	ST-361	IncFII, IncY, IncFIA, IncI1	*tet(A), sul1, bla*$_{EC}$*, bla*$_{CMY-145}$*, bla*$_{OXA-1}$*, catA1, aadA11, aadA2, aadA1, bla*$_{NDM-5}$*, mph(A), dfrA12, qepA8*	*capU, gad, sitA, terC, traT*
PK-5198	ST-167	IncFIA, IncFIC, ColKP3, ColRNAI, Col(BS512)	*tet(34), tet(A), sul1, bla*$_{EC-15}$*, bla*$_{CTX-M-15}$*, bla*$_{OXA-181}$*, bla*$_{TEM-1}$*, bla*$_{OXA-1}$*, catB3, aadA2, aac(6')-Ib-cr, bla*$_{NDM-5}$*, mph(A), dfrA12, ere(A), qnrS1*	*capU, fyuA, gad, irp2, iss, terC, traT*

PK-5202	ST-167	ColRNAI, IncFIA, IncFIC	*tet(34), sul1, sul2, bla*$_{EC-15}$, *bla*$_{CTX-M-15}$, *aadA2, aph(6)-Id, aph(3")-Ib, bla*$_{NDM-5}$, *dfrA12*	*capU, fyuA, gad, hra, irp2, terC, traT*
PK-5209	ST-361	IncI1, IncFII	*tet(A), bla*$_{EC}$, *bla*$_{OXA-1}$, *catA1, aadA1, bla*$_{NDM-21}$, *qepA1, qepA6*	*capU, gad, sitA, terC, traT*
PK-5224	ST-2851	Col156, IncFIA, ColRNAI, IncFII	*tet(A), sul1, bla*$_{EC-15}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *aadA2, bla*$_{NDM-5}$, *dfrA12*	*celb, gad, hra, lpfA, terC, traT*
PK-5238	ST-2851	IncI1, IncFIA, IncFII, Col(MG828)	*tet(A), sul1, bla*$_{EC-15}$, *bla*$_{CTX-M-15}$, *bla*$_{CMY-42}$, *aadA2, bla*$_{NDM-5}$, *mph(A), dfrA12*	*gad, hra, lpfA, terC, traT*

681

682 **Table 2. Characteristics of *E. coli* strains harboring *bla*$_{NDM}$ gene**

Isolate ID	Plasmid	Plasmid Type	G+C content	Size (bp)
PK-5055	pPK-5055-*bla*$_{NDM-5}$	IncFII-IncFIA	53.8%	138,592
PK-5081	pPK-5081-*bla*$_{NDM-5}$	IncFII-IncFIA-IncFIB	51.5%	145,982
PK-5099	pPK-5099-*bla*$_{NDM-20}$	IncFIA	54.7%	45,613
PK-5112	pPK-5112-*bla*$_{NDM-5}$	ColKP3-IncFII-IncFIA	52.6%	160,549
PK-5144	pPK-5144-*bla*$_{NDM-5}$	IncFIB	52.4%	166,610
PK-5160	pPK-5160-*bla*$_{NDM-1}$	IncHI2	45.9%	238,344
PK-5171	pPK-5171-*bla*$_{NDM-5}$	IncFII-IncFIA-IncFIB	51.9%	171,801
PK-5172	pPK-5172-*bla*$_{NDM-5}$	IncFII	51.6%	126,392
PK-5209	pPK-5209-*bla*$_{NDM-21}$	IncFII-IncFIA	52.9%	121,303

PK-5224	pPK-5224-*bla*$_{\text{NDM-5}}$	IncFII-IncFIA	52.7%	102,257	

IncFII
IncFIA
IncFIB
IncFIC
IncI1
IncY
IncX3
IncR

Reference
PK-5160
PK-5095
PK-5093
PK-5152
PK-5052
PK-5144
PK-5141
PK-5140
PK-5179
PK-5178
PK-5081
PK-5136
PK-5138
PK-5127
PK-5238
PK-5224
PK-5209
PK-5068
PK-5034
PK-5027
PK-5196
PK-5172
PK-5099
PK-5151
PK-5198
PK-5112
PK-5055
PK-5037
PK-5202
PK-5096
PK-5171
PK-5092
PK-5176
PK-5116

Sequence Types

- ST-156
- ST-2851
- ST-361
- ST-2450
- Unknown
- ST-167
- ST-1702
- ST-405

Inc Types of plasmids

- 0
- 0.1
- 0.2
- 0.3
- 0.4
- 0.5
- 0.6
- 0.7
- 0.8
- 0.9
- 1

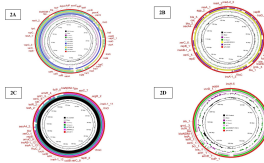

Figure 2. (A, B, C, and D). Circular comparison of different IncF plasmids bearing bla_{OXA} gene

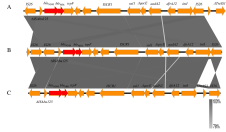

Figure 3. Three (A–C) major types of bla_{NDM}-bearing genetic contexts among the bla_{NDM}-bearing IncF-type plasmids

Figure 4. Genetic elements surrounding the blo_{NM4} gene on IncHI2 plasmid

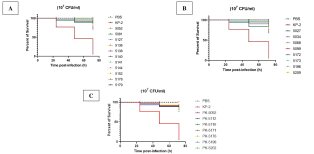

Figure 5. Kaplan-Meier curves A, B, and C showing the percentage of *G. mellonella* survival for 72 h post-infection with carbapenem-resistant strains of *E. coli* belonging to ST-405, ST-167, and ST-361, respectively