Emergence of Almost Identical F36:A-:B32 Plasmids Carrying blaNDM-5 and qepA in Escherichia coli from Both Pakistan and Canada

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Abstract: The New Delhi Metallo-β-lactamase (NDM) producing Enterobacteriaceae is spreading worldwide. Although the blaNDM gene has been identified in animal associated Enterobacteriaceae isolates in many countries, little is known about its occurrence in animal products in Pakistan. In this study, 13 Escherichia coli isolates were collected from chicken meat samples in Pakistan. Two isolates, 15978 and C4109, exhibited reduced susceptibility (MIC ≥1 μg/mL) to imipenem, and carried blaNDM-5 and blaNDM-7 gene, respectively. Whole-genome sequencing and Oxford Nanopore MinION sequencing revealed that 15978 and C4109 belonged to ST156 and ST167, respectively. blaNDM-7 was carried by an IncX3 plasmid that has disseminated worldwide, whereas blaNDM-5 was located on an F36: A-: B32 plasmid, which shared high identity with two plasmids carried by E. coli isolates from other countries (one from a patient in Canada). To the best of our knowledge, this is the first report characterizing blaNDM-carrying plasmids from chicken meat samples in Pakistan. The dissemination of almost identical blaNDM-5-bearing F36:A-:B32 and blaNDM-7-bearing IncX3 plasmids in different countries highlights the importance of international trade and travel in the spread of antimicrobial resistance strains and plasmids worldwide.

Keywords: plasmid, animal food, carbapenemase, blaNDM

Introduction

Carbapenems are last-resort drugs for treating infections caused by multidrug-resistant (MDR) bacteria. However, resistance to carbapenems in gram-negative bacteria, especially Enterobacteriaceae, has increased rapidly over the last decade and poses an increasing threat to global public health.1,2 Carbapenem resistance in Enterobacteriaceae is primarily attributed to carbapenemase enzymes, especially Klebsiella pneumoniae carbapenemase (KPC) and the New Delhi metallo-β-lactamase (NDM).3 blaNDM was firstly discovered from a Swedish patient in India during 2007.4 Since then, it has been increasingly identified throughout the world and was found epidemic in the Indian subcontinent including Pakistan, Afghanistan, and the Balkans regions etc.5 NDM is able to hydrolyze almost all β-lactams, and the hydrolytic activity of NDM enzymes cannot be weakened by β-lactamase inhibitors, such as clavulanate, tazobactam, sulbactam, and avibactam, leaving limited therapeutic options for infections caused by NDM-producing Enterobacteriaceae.6 blaNDM genes are usually located on plasmids capable of efficient transfer between bacterial species and hosts in and out of hospitals.5

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Though carbapenems are not legally prescribed for use in livestock production, the occurrence of carbapenem-resistant Enterobacteriaceae (CRE), especially NDM-producing Enterobacteriaceae has been increasingly reported in livestock and meat products in the world. Yet, systematic study on the prevalence and characterization of NDM-producing Enterobacteriaceae from food animals and animal-derived foods remain to be sporadic in Pakistan. Here, for the first time, we characterized two NDM-producing Escherichia coli strains that were recovered from retail chicken meat samples in Pakistan.

Materials and Methods

Bacterial Isolation, Antimicrobial Susceptibility Testing, and Detection of the blaNDM Gene

In March 2018, fourteen chicken meat samples were collected from local broiler meat outlets in Faisalabad, Pakistan. Antibiotic-free MacConkey agar plates were used to isolate E. coli strains. The isolates were identified by MALDI-TOF MS (Shimadzu-Biotech Corp., Kyoto, Japan).

The MICs of 14 antimicrobial agents, including ampicillin, cefotaxime, ceftazidine, cefoxitin, florfenicol, fosfomycin, streptomycin, doxycycline, ciprofloxacin, imipenem, colistin, amikacin, gentamycin, and tigecycline, were assessed by either agar dilution or broth microdilution method (colistin and tigecycline) with the E. coli strain ATCC 25922 as the control according to CLSI guideline. Isolates that showed reduced susceptibility to imipenem (MIC ≥1 μg/mL) were selected for PCR screening of carbapenemase genes. PCR products were confirmed by sequencing.

Whole Genome Sequencing (WGS)

Whole genome DNA of NDM-producing E. coli isolates, 15978 and C4109, was extracted and sequenced using HiSeq (Illumina, San Diego, CA, USA) platforms. Afterwards, SOAPdenovo (version 2.04) was used to assemble sequence reads into contigs and to extract blaNDM-bearing plasmid contigs. To obtain the complete sequence of the blaNDM-containing plasmid, we then sequenced E. coli 15978 on Oxford Nanopore MinION. The assemblies of long Nanopore reads and the short Illumina reads were combined via Unicycler version 0.4.3. The resistance genes, chromosomal mutations, virulence genes, plasmid type, and MLST of the two blaNDM positive strains were analyzed by ResFinder 3.2, PointFinder, VirulenceFinder, PlasmidFinder, and MLST (https://cge.cbs.dtu.dk/services/), respectively. Comparative analysis of blaNDM-carrying plasmids was carried out using BLAST tools and BLAST Ring Image Generator (BRIG).

Results and Discussion

A total of 13 E. coli isolates were recovered from 13 retail chicken meat samples. Two isolates, 15978 and C4109, showed reduced susceptibility to imipenem (MIC ≥1 μg/mL), and were identified to carry blaNDM-5 and blaNDM-7, respectively (Table 1). The two isolates showed resistance to cefotaxime, ceftazidine, and cefoxitin, but remained susceptible to fosfomycin, colistin, amikacin, gentamycin, and tigecycline (Table 1). In addition, C4109 exhibited resistance to streptomycin and ciprofloxacin, while 15978 showed resistance to doxycycline and ciprofloxacin. blaNDM-5 and blaNDM-7 were successfully transferred to recipients E. coli C600 or E. coli DH5α by conjugation and transformation, respectively.

WGS showed that E. coli 15978 and C4109 belonged to ST156 and ST167, respectively (Table 1). These two E. coli sequence types were also related to blaNDM dissemination worldwide in humans, animals, and food. The resistance genes, chromosomal mutations, and virulence genes of the two isolates were displayed in Table 1. It showed that 15978 and C4109 carried eight and five other resistance genes, respectively.

In E. coli C4109, blaNDM-7 was carried by a 49,828-bp IncX3 plasmid pHN4109c (MK088485), which shared 92% coverage and 99% identity with pKW537-NDM (KX214669) from clinical E. coli in Kuwait, pNDM5_IncX3 (KU761328) from clinical K. pneumoniae in China, and vig0000260 (CP021738) from E. coli in USA (Figure 1A). IncX3 has dominated the spread of blaNDM in many countries, particularly in Asian countries, such as China, Korea, Myanmar, and India. To the best of our knowledge, this study was the first to identify blaNDM-positive IncX3 plasmid in Pakistan. Similar to other reports, blaNDM-7 was embedded in an IS26-blaNDM-ΔTn2 transposition unit which inserted into umuD gene in pHN4109c. However, a 3664-bp transposon Tn5403 was inserted in the IS3000 gene, which formed a unique genetic structure together with 5-bp direct repeats (TACAT) (Figure 1B).

The blaNDM-5-carrying plasmid pHN15978 (MK291500) was a 128,762-bp F36:A-:B32 plasmid containing 151 ORFs. It was comprised of a typical IncF-type backbone, encoding genes for replication, transfer, maintenance, stability functions, and a multidrug resistance region of 28145-bp. BLAST homology analysis demonstrated that the sequence of pHN15978 showed 99% identity and 100% query coverage with E. coli strain AR_452 plasmid unnamed1 (CP030329.1) and
FDAARGOS_448 plasmid unnamed1 (CP023959.1), with only 3-bp and 5-bp nucleotide differences, respectively (Figure 1B). E. coli AR_452, with an unknown geographic origin, was from human and retained in the CDC & FDA Antibiotic Resistance (AR) Isolate Bank (https://www.cdc.gov/drugresistance/resistance-bank/). FDAARGOS_448 was isolated from a patient in Canada in August 2014 and was stored in Database for Reference Grade Microbial Sequences (FDA-ARGOS) database (https://www.fda.gov/MedicalDevices/ScienceandResearch/DatabaseforReferenceGradeMicrobialSequences/default.htm). Of note, E. coli AR_452 was also assigned to ST156 and FDAARGOS_448 belonged to ST405. It seemed that blaNDM-5 gene might be circulating among human and food by E. coli ST156 clones or pHN15978-like plasmids in different regions. Unlike epidemic IncX3 plasmids, F36:A:-B32 plasmid is less related to the spread of blaNDM and this is the first time to report blaNDM-positive F36:A:-B32 plasmid. Thus, the identification of almost identical F36:A:-B32 plasmids carrying blaNDM-5 in geographically far away countries, Pakistan and Canada, is surprising. Though there is no clear epidemiological link between E. coli 15978, E. coli strain AR_452, and FDAARGOS_448, poultry trade between Pakistan and Canada might partly explain these findings considering the fact that Pakistan poultry industry was built with the help of Canada based company shaver poultry breeding farms in 1962. In addition, international travel and migratory birds might also be responsible for the global dissemination of this F36:A:-B32 plasmid.23,24

The multidrug resistance region of pHN15978 was mainly composed of three mobile modules (Figure 1C). The first part harbored β-lactam and macrolide resistance genes. More specifically, it consisted of a derivative of ΔTn2 (blaTEM-1) and an IS26-mph(A)-mrx-mphR(A)-IS6100 unit. The resistance region was also identified in E. coli plasmid pCARB35_02 (CP031655.1, dog, UK) and K. pneumoniae plasmid pCRKP-1215_2 (CP024840.1, human, Korea). In the second part, blaNDM-5 gene was found embedded in an ISCR1 complex.

### Table 1 Characterization of NDM-Producing Escherichia coli Isolates and Transconjugant or Transformant

| Strain            | C4109   | C4109 Transformant | E. coli DH5α | 15978 | 15978 Transconjugant | E. coli C600 |
|-------------------|---------|--------------------|--------------|-------|----------------------|--------------|
| Ampicillin        | >128    | >128               | 8            | >128  | >128                 | 4            |
| Cefotaxime        | >128    | 64                 | 0.03         | >128  | 32                   | 0.125        |
| Cefazidine        | >128    | >128               | 0.125        | >128  | >128                 | 0.06         |
| Cefoxitin         | >128    | >128               | 2            | >128  | 128                  | 4            |
| Florfenicol       | 16      | 2                  | 1            | 16    | 2                    | 2            |
| Fosfomycin        | 32      | 8                  | 2            | 32    | 4                    | 2            |
| Streptomycin      | 256     | >128               | 1            | 8     | >128                 | >128         |
| Doxycycline       | 4       | 1                  | 0.125        | 128   | 128                  | 0.5          |
| Ciprofloxacin     | >64     | 0.008              | 0.002        | >64   | 0.125                | 0.008        |
| Imipenem          | 4       | 2                  | 0.125        | 1     | 1                    | 0.125        |
| Resistance genes  | blaNDM-5, blaCTX-M-15, aph(3′)-Ib, aph(6)-Id, qnrS1, sul1 | blaNDM-7 | - | blaNDM-5, blaTEM-1b, aadA2, qepA, mph(A), mphR(A), sul1, tet(B), dfrA12 | - |
| Chromosomal point mutations | GyrA: S83L, D87N, ParE: S458A, ParC: S80I | GyrA: S83L, D87N | - | GyrA: S83L, D87N, ParC: S80I, EB4G |
| Virulence genes   | capU, iss | gas, iss, lpfA | - | - | - |

Note: All isolates were susceptible to colistin, amikacin, gentamycin, and tigecycline.
class 1 integron, which was sequentially organized as IS26-ΔISaba125-bla_{NDM-5}-ble_{MBL}-trpF-tet-ISCRI-qacEΔ1-sul1-aad2-hp-dfrA12-Δallback, which was identical with the E. coli plasmid pM309-NDM5 (F36:A-:B32) from E. coli strain from mankind in Kuwait, pNDM-MGR194 (KF220657.1) from K. pneumoniae strain from human in India, pNDM5_IncX3 (KCU766132.1) from K. pneumoniae strain from human in China, pOM26-1 (KP776609.1) from E. coli strain from human in Oman, pNDM-NDM5 (MG823582) from E. coli strain from human in China, and por00000260 (CP025383.1) from E. coli strain from USA, respectively. The third part only contained one resistance gene, qepA. This genetic structure was sequentially organized as groEL/Δin1I-ISCRI-qepA-Δin1I-Δin2-ΔIS1, which was identical with the E. coli plasmid pM309-NDM5 (F36:A4-B- or F36:A20-B-, AP018833.1), pNDM-d2e9 (F2:A-2-B-), CP026201.1, and pAMA167-NDM-5 (F1:A1:B49, CP024805.1) from Khan, USA, and Denmark, respectively.21,25 The third part only contained one resistance gene, qepA. This genetic structure was sequentially organized as groEL/Δin1I-ISCRI-qepA-Δin1I-Δin2-ΔIS1, which was highly similar to that of pHN3A11 (JX997935.2, E. coli, cat, China),26 pMH16-367M_1 DNA (AP018565.1, Morganella morgani, human, Vietnam), and pJJ1887-5 (CP014320.1, E. coli, human, USA).27

**Conclusion**

In summary, we firstly characterized two bla_{NDM-5}-carrying plasmids from chicken meat samples in Pakistan. The dissemination of almost identical bla_{NDM-5}-bearing F36:A-:B32 plasmids and bla_{NDM-7}-bearing IncX3 plasmids in different countries highlights the importance of international trade and travel in the spread of antimicrobial resistance strains and plasmids worldwide.

**Accession Number**

The complete nucleotide sequence of plasmid pHN4109c and pHN15978 have been deposited in GenBank under accession no. MK088485 and MK291500, respectively.

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**Disclosure**

The author reports no conflicts of interest in this work.
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