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SHORT REPORT

Emergence of Almost Identical F36:A-:B32 Plasmids Carrying *bla*$_{NDM-5}$ 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 *bla*$_{NDM}$ 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 *bla*$_{NDM-5}$ and *bla*$_{NDM-7}$ gene, respectively. Whole-genome sequencing and Oxford Nanopore MinION sequencing revealed that 15978 and C4109 belonged to ST156 and ST167, respectively. *bla*$_{NDM-7}$ was carried by an IncX3 plasmid that has disseminated worldwide, whereas *bla*$_{NDM-5}$ 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 *bla*$_{NDM}$-carrying plasmids from chicken meat samples in Pakistan. The dissemination of almost identical *bla*$_{NDM-5}$-bearing F36:A-:B32 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.

Keywords: plasmid, animal food, carbapenemase, *bla*$_{NDM}$

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] *bla*$_{NDM}$ 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] *bla*$_{NDM}$ genes are usually located on plasmids capable of efficient transfer between bacterial species and hosts in and out of hospitals.[3]

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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.[7–9] 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.[10] 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 bla_{NDM} 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.[11] Isolates that showed reduced susceptibility to imipenem (MIC ≥1 μg/mL) were selected for PCR screening of carbapenemase genes.[12] 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 bla_{NDM}-bearing plasmid contigs. To obtain the complete sequence of the bla_{NDM-5}-carrying 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.[13] The resistance genes, chromosomal mutations, virulence genes, plasmid type, and MLST of the two bla_{NDM} positive strains were analyzed by ResFinder 3.2, PointFinder, VirulenceFinder, PlasmidFinder, and MLST (https://cge.cbs.dtu.dk/services/), respectively. Comparative analysis of bla_{NDM}-carrying plasmids was carried out using BLAST tools and BLAST Ring Image Generator (BRIG).[14]

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 bla_{NDM-5} and bla_{NDM-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. bla_{NDM-5} and bla_{NDM-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 bla_{NDM} dissemination worldwide in humans,[15,16] animals,[17,18] and food.[19] 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, bla_{NDM-7} was carried by a 49,828-bp IncX3 plasmid pHN4109c (MK088485), which shared 92% coverage and 99% identity with pKW53T-NDM (KX214669) from clinical *E. coli* in Kuwait, pNDM5_IncX3 (KU761328) from clinical *K. pneumoniaee* in China, and tig00000260 (CP021738) from *E. coli* in USA (Figure 1A). IncX3 has dominated the spread of bla_{NDM} in many countries, particularly in Asian countries, such as China,[9] Korea,[20] Myanmar,[21] and India.[22] To the best of our knowledge, this study was the first to identify bla_{NDM}-positive IncX3 plasmid in Pakistan. Similar to other reports, bla_{NDM-7} was embedded in an IS*26*-bla_{NDM}-ΔTn*2* transposition unit which inserted into *umuD* gene in pHN4109c. However, a 3664-bp transposon Tn*5403* was inserted in the IS*3000* gene, which formed a unique genetic structure together with 5-bp direct repeats (TACAT) (Figure 1B).

The bla_{NDM-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

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
ST	ST167			ST156		
Ampicillin	>128	>128	8	>128	>128	4
Cefotaxime	>128	64	0.03	>128	32	0.125
Ceftazidine	>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	*bla*$_{NDM-7}$, *bla*$_{CTX-M-15}$, *aph(3")-Ib, aph(6)-Id, qnrS1, sul1*	*bla*$_{NDM-7}$	-	*bla*$_{NDM-5}$, *bla*$_{TEM-1b}$, *aadA2, qepA, mph(A), mdf(A), sul1, tet(B), dfrA12*	*bla*$_{NDM-5}$, *bla*$_{TEM-1b}$, *aadA2, qepA, mph(A), sul1, tet(B), dfrA12*	-
Chromosomal point mutations	GyrA: S83L, D87N ParE: S458A ParC: S80I			GyrA: S83L, D87N ParC: S80I, E84G		
Virulence genes	*capU, iss*			*gad, iss, lpfA*		

Note: All isolates were susceptible to colistin, amikacin, gentamycin, and tigecycline.

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 *bla*$_{NDM-5}$ 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 *bla*$_{NDM}$ and this is the first time to report *bla*$_{NDM}$-positive F36:A-:B32 plasmid. Thus, the identification of almost identical F36:A-:B32 plasmids carrying *bla*$_{NDM-5}$ 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 ΔTn*2* (*bla*$_{TEM-1}$) and an IS*26-mph(A)-mrx-mphR(A)*-IS*6100* unit. The resistance region was also identified in *E. coli* plasmid pCARB35_02 (CP031655.1, dog, UK) and *K. pneumoniaee* plasmid pCRKP-1215_2 (CP024840.1, human, Korea). In the second part, *bla*$_{NDM-5}$ gene was found embedded in an IS*CR1* complex

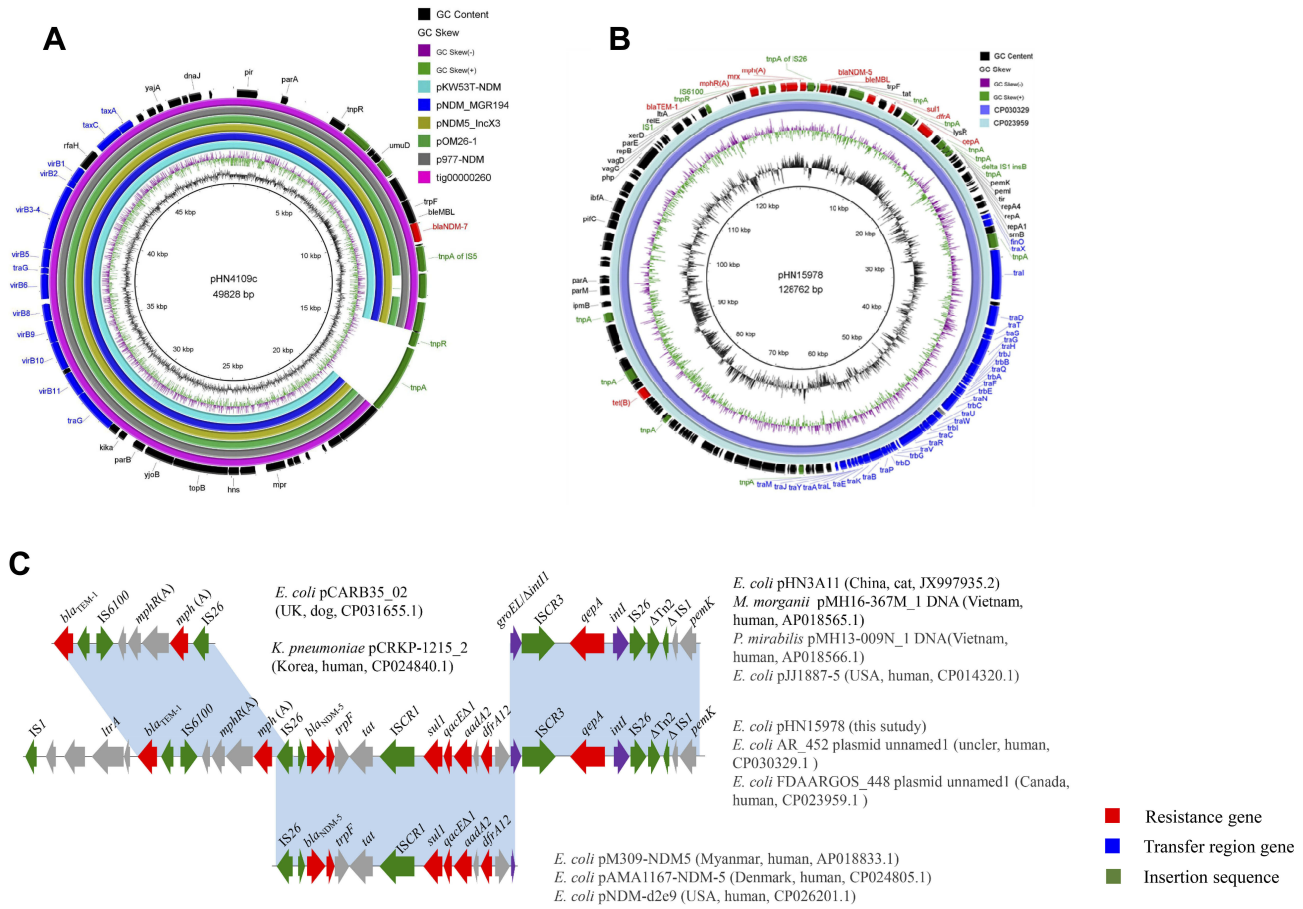

Figure 1 Comparisons of *bla*$_{NDM}$-positive plasmids and multidrug resistance region of pHN15978. (**A**) comparisons of *bla*$_{NDM}$-positive IncX3 plasmids. The innermost ring was the reference plasmid pHN4109c. The light blue, dark blue, yellow, green, grey and pink ring represents pKW53T-NDM (KX214669) from *E. coli* strain from human in Kuwait, pNDM_MGR194 (KF220657.1) from *K. pneumonia* strain from human in India, pNDM5_IncX3 (KU761328) from *K. pneumonia* strain from human in China, pOM26-1(KP776609.1) from *E. coli* strain from human in Oman, p977-NDM (MG825382) from *E. coli* strain from pork in China, and tig00000260 (CP021738) from *E. coli* strain from USA, respectively. (**B**) comparisons of *bla*$_{NDM-5}$-positive F36:A-:B32 plasmids. The innermost ring was the reference plasmid pHN15978 (128762bp, chicken meat, Pakistan, MK291500). The light blue and light green ring represents *E. coli* strain AR_452 plasmid unnamed1 (128762bp, CP030329) and *E. coli* strain FDAARGOS_448 plasmid unnamed1 (128761bp, CP023959) from Canada (human urine, 2014.8), respectively. (**C**) Comparisons of multidrug resistance region of pHN15978. Regions of ≥99.0% nucleotide sequence identity are shaded grayish blue.

class 1 integron, which was sequentially organized as IS*26*-ΔIS*Aba125*-*bla*$_{NDM-5}$-*ble*$_{MBL}$-*trpF*-*tat*-IS*CR1*-*qacE*Δ*1*-*sul1*-*aad2*-*hp*-*dfrA12*-Δ*intI1*, which was identical with the *E. coli* plasmid pM309-NDM5 (F36:A4:B- or F36:A20:B-, AP01 8833.1), pNDM-d2e9 (F2:A-:B-, CP026201.1), and pAMA1 167-NDM-5 (F1:A1:B49, CP024805.1) from Myanmar, USA, and Denmark, respectively.[21,25] The third part only contained one resistance gene, *qepA*. This genetic structure was sequentially organized as *groEL*/Δ*intI1*-IS*CR3*-*qepA*-Δ*intI1*-IS*26*-ΔTn*2*-ΔIS*1*, and was highly similar to that of pHN3A11 (JX997935.2, *E. coli*, cat, China),[26] pMH16-367M_1 DNA (AP018565.1, *Morganella morganii*, human, Vietnam), and pJJ1887-5 (CP014320.1, *E. coli*, human, USA).[27]

Conclusion

In summary, we firstly characterized two *bla*$_{NDM}$-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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