

Genomic characterization of carbapenem resistant *Escherichia coli* from multiple hospitals in Nanjing, China: focusing on frequent co-occurrence of blaNDM and blaKPC-2

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Research

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Abstract

Background

The increasing emergence of carbapenem resistant *Escherichia coli* (CREC) poses a potential threat to public health, hence genomic characterization of isolates is needed for a better understanding of its transmission and implementation of infection control measures.

Materials and methods

Eleven CREC isolates were collected in 2015 from 6 hospitals in Nanjing, China, and analyzed using whole genome sequencing. Resistance determinants, virulence elements, multi-locus sequence type (MLST), serotypes, phylogeny and fimH types were determined.

Results

All of the CREC carried at least one carbapenemase. NDM-5 (n = 9) was the most frequent carbapenemase, followed by KPC-2 (n = 3) and NDM-1 (n = 2); three isolates produced NDM-5 and KPC-2. Ten out of the 11 isolates co-carried *bla*CTX-M variants. MLST analysis found 7 distinct STs, including ST410 (n = 2), ST3489 (n = 1), ST156 (n = 1), ST683 (n = 1), ST297 (n = 1), ST167 (n = 1), and ST361 (n = 1). Six distinct serotypes and 8 Fim types were identified. A great diversity of plasmid profiles was observed with plasmid replicon IncX3 being the most frequent (n = 11). Phylogenetic analysis showed great diversity between the 11 CREC isolates and also between 6 additional isolates co-carrying *bla*NDM and *bla*KPC which were selected from the strains collection of Nanjing Drum Tower Hospital for comparison. Conjugation assays demonstrated that *bla*NDM was transferable.

Conclusion

NDM is the major carbapenemase among CREC, with NDM-5 being the main variant which can be horizontally disseminated by IncX3 plasmids. These isolates displayed genetic diversity by MLST, Fim typing and serotyping. We herein provided the first report on emergence of NDM-5 producing *E. coli* ST297, ST683, ST3489, and NDM-1 producing *E. coli* ST361.

Introduction

Escherichia coli mainly inhabits the lower intestinal tract of warm-blooded animals. It is a major pathogen for numerous types of infections, such as intestinal, urinary, and respiratory tract infections in humans and other animals (1). In recent years, carbapenems have been increasingly used as the most effective antibiotic in clinical therapy for infections caused by multidrug-resistant (MDR) strains, due to production of extended spectrum β -lactamases (ESBLs) or AmpC-type β -lactamases(2). Therefore, the

frequent occurrence of carbapenem-resistant *Escherichia coli* (CREC) worldwide has been posing a threat to public health (3, 4).

Production of carbapenemases has been so far the main mechanism for carbapenem resistance in *Enterobacterales*(5), and New Delhi metallo- β -lactamase (NDM) is the major carbapenem resistance gene in *E. coli* all over the world (6). It is worthy to note that the co-occurrence of multiple β -lactamases among single bacteria species (7), especially carbapenemase, such as co-production of NDM-1 and *Klebsiella pneumoniae* carbapenemase 2 (KPC-2)(8), co-occurrence of KPC-2 and OXA-48, have so far been frequently described in multiple clinical *Enterobacterales*, such as *K. pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii*(8-10), indicating that the production of carbapenemase has become an urgent public health threat.

Multiple studies have shown that that *bla*NDM is often located on IncX3 plasmids, (11, 12), but little information on virulence genes, serotyping and fim typing is available for such strains.

In this study, 11 CREC strains collected from 6 hospitals in Nanjing were subjected to next-generation sequencing, and genomic epidemiology including resistance determinants, virulence factors, serotyping, fim typing and plasmid replicons were further analyzed. Based on the high prevalence of strains co-producing NDM-5 and KPC-2, 43 additional CREC were retrieved from a tertiary hospital during 2013-2017 and further screened for co-production of KPC and NDM. Pulsed-Field Gel Electrophoresis (PFGE) was further performed to analyze the genetic relatedness, and conjugation assays were implemented to determine the transferability of the plasmids.

Materials And Methods

Isolates collections

Firstly, eleven CREC isolates were collected from 6 hospitals during 2015 June-2015 December in Nanjing city. These strains were isolated from urine (n=4), sputum (n=3), blood (n=1), bile (n=1), secretion of uterus neck (n=1), and the source of one strain was unknown.

A total of 43 consecutive non-duplicate isolates collected in Nanjing Drum Tower hospital during 2013-2017 were further analyzed for isolates co-producing KPC-2 and NDM. Among them, 4 strains were isolated in 2013, 10 in 2014, 2 in 2015, and 11 from 2016 and 16 in 2017. The source of the samples was as follows: urine (n=18), blood (n=9), sputum (n=6), secretion (n=3), bile (n=3), abdominal dropsy (n=2), and pus (n=1).

All the CREC strains were identified by Vitek 2.0 (BioMérieux. Marcy l'Etoile, France) or ATB 32E Semi-auto identification machine (Bio-

Mérieux, France). Isolates resistant to at least one carbapenem (imipenem, meropenem, ertapenem) were included in the study.

DNA Extraction

The Ultraclean Microbial DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, US) was used to extract genomic DNA. The NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used for measuring the DNA concentration and purity for whole genome sequencing.

Whole Genome Sequencing, denovo Assembly, Scaffolding, and Annotation

The prepared pair-end DNA library was sequenced on the MiSeq (Illumina, SanDiego, CA, USA). Denovo assembly of the paired-end reads was performed by CLC Genomics Workbench v7.0.4 (QIAGEN, Hilden, Germany) after quality trimming ($Q_s \geq 20$). Scaffolding was finished using SSPACE standard version 3.0 and the gaps within scaffolds were further closed by GapFiller (13, 14). Then genomes were then submitted to NCBI for annotation.

Analysis of genomic epidemiology

The antimicrobial resistance determinants and virulence factors were identified using Resfinder v2.1 (<http://cge.cbs.dtu.dk/services/ResFinder-2.1/>) and Virulence Finder 2.0 (<https://cge.cbs.dtu.dk/services/VirulenceFinder/>); the 11 CREC were typed by multi-locus sequence typing (MLST) 2.0 (<https://cge.cbs.dtu.dk/services/MLST/>), Plasmid Finder 2.1 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>), Serotype Finder 2.0 (<https://cge.cbs.dtu.dk/services/SerotypeFinder/>), and FimTyper 1.0 (<https://cge.cbs.dtu.dk/services/FimTyper/>).

Phylogenetic relationship of 11 CREC

The core-genome phylogeny of the 11 CREC was constructed by using single-nucleotide polymorphisms (SNP)-sites detected from 1010 core genes (identity > 95%; coverage = 100%) derived from 502 ST11 strains (15). A maximum-likelihood tree was calculated using RAxML version 8.2.8, with general time-reversible model and 100 bootstraps (16). Interactive Tree Of Life (<https://itol.embl.de>) was used to produce the phylogenetic tree (17).

The screening of *Escherichia coli* co-producing NDM and KPC-2 carbapenemases

A total of 43 CREC strains collected from our hospital during 2013-2017 were further analyzed for the prevalence of *E. coli* co-producing NDM-5 and KPC-2. Presence of genes encoding carbapenemases (*blaKPC*, *blaAIM*, *blaSIM*, *blaSPM*, *blaVIM*, *blaIMP*, *blaOXA*, *blaDIM*, *blaNDM*, *blaGIM*, and *blaGES*) was analyzed by PCR and DNA sequencing (18).

Pulsed-field gel electrophoresis

Isolates selected from the 43 CREC were further analyzed for genetic relatedness by PFGE, which was performed according to the protocol as previously described (19). Briefly, fresh colonies were mixed with proteinase K (Merck Sharp & Dohme Ltd, Germany) into plugs. After the plugs were digested by restriction

endonuclease XbaI (Fermentas, ABI, Germany), the resultant DNA fragments were separated in a PFGE CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA) in 0.5×Tris-borate-EDTA buffer at 120 V for 19 h. The pulse times ranged from 2.2 s to 54.2 s. Finally, the BioNumerics software (Applied Math, Sint-Matens-Latem, Belgium) was used to analyze the banding patterns.

Conjugation assay

For the 6 isolates co-carrying *bla*KPC and *bla*NDM, broth mating was performed in order to analyze the transferability of these genes according to the protocol prescribed previously(20). Azide resistant *E. coli* J53 was used as the recipient. Briefly, fresh colonies were inoculated into 5 ml LB broth and incubated at 37°C, 200 rpm. After 5 hours, 500 µl recipient cells and 100 µl donor were suspended in 5 ml LB broth for overnight culture at 37°C, 200 rpm, then 100 µl were plated onto the LB plates containing 30 mg/L cefoxitin and 100 mg/L sodium azide for *E. coli* J53. PCR (amplification for *bla*NDM and *bla*KPC) and Eric-PCR were used to verify conjugants.

Nucleotide Sequence Genbank Accession Numbers

The Whole Genome Shotgun BioProject for these CREC isolates has been deposited at GenBank, the accession number of each strain were as follows: JABEWI000000000(NJSETYY11), JABEWJ000000000 (NJSETYY14), JABEWL000000000 (NJLSYY40), JABEWM000000000 (NJSDYYY42), JABEWN000000000 (NJJYYY51), RZMM000000000 (NJGLYY3940), RZMI000000000 (NJGLYY3610), JABEWH000000000 (NJMJYY4), JABEWK000000000 (NJSFYBJY17).

Results

Genomic epidemiology of 11 CREC

Genomic analysis showed that all the 11 CREC strains carried NDM carbapenemases, including 2 NDM-1 and 9 NDM-5, while three strains co-carried NDM-5 and KPC-2; ten out of the 11 isolates carried CTX-M variants, including CTX-M-55 (n=4), CTX-M-65 (n=2), CTX-M-14 (n=2) and CTX-M-15 (n=2). AmpC enzyme including 2 CMY-2 and 3 CMY-43, plasmid mediated quinolone resistance (PMQRs), including *oqxAB* (n=2), *qnrA1* (n=1), *qnrS1* (n=4), *aac(6')Ib-cr* (n=4), *qepA* (n=1), and 4 *fosA3* were also identified. Virulence genes analysis showed that 12 VFs were detected, with *gad* (n=9) being the most frequent one, followed by *lpfA* (n=7) and *iss* (n=7), other VFs including *astA* (n=3), *cma* (n=3), *capU* (n=3), *iroN* (n=2), *sat* (n=1), *senB* (n=1), *sepA* (n=1), *iha* (n=1) and *cnf1* (n=1) were also found.

MLST analysis found 7 distinct STs, including ST410 (N=2), ST3489 (n=1), ST156 (n=1), ST683 (n=1), ST297 (n=1), ST167 (n=1) and ST361 (n=1), in addition, 3 new STs were identified;

The 11 strains were typed into 5 *E. coli* O groups (O8, O9, O25, O89, O121, O3) and 8 H groups (H9, H10, H4, H21, H40, H45, H30 and H26). Six distinct serotypes were found including O8:H9 (n=3), O9:H4 (n=1), O89:H9 (n=1), O30:H21 (n=1), O25:H26 (n=1) and O9:H30 (n=1).

Analysis of FimH identified 8 types, including Fim24 (n=2), Fim23 (n=2), Fim34 (n=1), Fim38 (n=1), Fim121 (n=1), Fim276 (n=1), Fim54 (n=1) and Fim31 (n=1).

A great diversity of plasmid profiles was observed. Among them, plasmid replicon IncX3 was found among all the 11 strains followed by IncFII (n=7) and IncFIB (n=6), additionally, IncI1 (n=3), IncFIA (n=3), IncY (n=2) and IncFIC (n=2) were also detected.

Phylogenetic characterization of 11 CREC strains

The phylogenetic tree showed that 11 CREC evolve into 2 main clades albeit a great diversity was observed. Two isolates from Nanjing Children's hospital were likely clonal.

Prevalence of isolates co-producing NDM-5 and KPC-2

Among the 43 CREC collected from our hospital during 2013-2017, 6 KPC-2 and 23 NDM were identified, two strains co-producing NDM-5 and KPC-2 and one strain co-carrying NDM-1 and KPC-2 were found. One was isolated from the urine of an inpatient in ICU in 2014, the other two strains were isolated from abdominal dropsy and sputum of the different patients in 2017.

Genetic relatedness of strains co-carrying *blaKPC* and *blaNDM*

PFGE displayed a high diversity of the 6 strains co-producing *blaKPC* and *blaNDM*, indicating that these strains were not from the same clone.

Transferability of *blaKPC* and *blaNDM*

Conjugation assay revealed that the *blaNDM* of all the 6 isolates was transferable to *E. coli* J53. However, we could not isolate any conjugants with *blaKPC*, suggesting that the *blaKPC* and *blaNDM* were not on the same plasmid.

Discussion

In this study, we provided data on genomic epidemiology of 11 CREC strains from 6 hospitals in Nanjing, Jiangsu province. Based on the high co-occurrence of *blaNDM-5* and *blaKPC-2*, 43 CREC strains collected from a tertiary hospital during 2013-2017 were further screened to investigate the prevalence of such strains in our hospital. This is the first study that provided the genomic epidemiology of the CREC from multiple hospitals in Nanjing.

The high prevalence of NDM among 11 CREC in our study is in accordance with the previous report (6), indicating that NDM is the major carbapenemase for carbapenem resistance in *E. coli*, which may result from the low fitness burden of the plasmid harboring *blaNDM* in *E. coli* (21). Moreover, we found a high co-occurrence of KPC-2 and NDM-5, 5 out of the 6 strains that were isolated from our hospital. Other resistance markers, *blaOXA-1*, *blaCMY*, *blaCTX-M*, and *fosA3*, *rmtB*, *qnrS* and *aac(6')-Ib-cr* were also found in these NDM-5 and/or KPC-2 producing strains.

Virulence gene analysis revealed several major VFs in CREC, among them, *gad* encodes glutamate decarboxylase, which is the structural component of the major acid resistance system that protects *E. coli* from strong acid stress (pH < 3), typically encountered in the mammalian gastrointestinal tract (22), *lpfA* (Long polar fimbriae) is a putative adhesion gene, encoding one of the few fimbrial adhesions of enterhemorrhagic *E. coli* O157:H7 associated with colonization on host intestine, which play essential roles during the bacterial infection process(22). *iss* (increased serum survival) is the most common avian pathogenic *E. coli* encoding gene. It has been identified as a virulence trait associated with the virulence of *E. coli*, causing colibacillosis in poultry (23). The high prevalence of these VFs among CREC may suggest that CREC mainly colonize the host intestine, and they might have a lower potential to cause human disease. Noteworthy, a strain isolated from urine belong to a new ST, which not only carried *gad* and *iss*, *lpfA*, but also *sat* (secreted autotransporter toxin), *senB* (Plasmid-encoded enterotoxin), *sepA* (Shigella extracellular protein A), *iha* (Adherence protein) and *cnf1* (Cytotoxic necrotizing factor). It is known that *sat* can promote cytotoxic effects in several lines of undifferentiated epithelial cells and is highly prevalent in certain *E. coli* pathogenic groups responsible for urinary and intestinal infections(24). Cnf1 is frequently expressed in clinical UPEC isolates, CNF1-producing and β -hemolytic *E. coli* strains most notably cause urinary tract and meningeal infections in humans(25). Altogether, the wide presence of these VFs among the urinary CREC may indicate a higher pathogenicity of this strain.

ST131 as a multidrug clone has spread extensively throughout the world (26). However, ST131 was not detected in our study. The multiple distinct STs identified in our study indicated the diversity of these CREC, which was also confirmed by the phylogenetic relationship. NDM-5 has been reported in *E. coli* ST410, ST156, and ST167 (20). However, to the best of our knowledge, NDM-5 producing *E. coli* ST297, ST683 and ST3489, as well as NDM-1 producing *E. coli* ST361 has not been reported previously.

O8:H9 as the most common serotype in our study was consistent with previous report, indicating that O8:H9 is a clinically-relevant serotype correlated with multidrug resistance(27)

Altogether, the multiple fimH types, the diverse STs and serotypes in our study, indicates a high diversity of these CREC strains. Although further PFGE from the strains co-carrying KPC and NDM excludes an epidemic dissemination of the frequent occurrence of these strains, the emergence of these strains pose a potential public health threat.

Multiple plasmid replicons were found among these CREC. However, plasmid replicon IncX3 detected among all the NDM-producing *E. coli* may provide evidence that IncX3 is the main host for NDM (28). Note worthily, we found a frequent co-occurrence of strains co-carrying *bla*NDM and *bla*KPC-2 among clinical CREC, albeit our further study demonstrated that NDM and KPC-2 were not harbored by the same plasmid.

In conclusion, genomic analysis found that NDM is the main carbapenemase for 11 CREC strains, with a frequent occurrence of KPC. These CREC strains displayed genetic diversity by MLST, Fim typing and serotyping, as well as the phylogenetic relationship. IncX3 may be the main plasmid for NDM, and *gad*, *iss* and *lpfA* were the main VFs; Albeit a frequent occurrence of strains co-carrying *bla*NDM and *bla*KPC-2

were detected. No clonal dissemination were found; based on the potential public health threat posed by these strains, infection control measures should be further strengthened.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

CXL performed genome extraction and data analysis ; ZJ implemented strains collection; CL, ZWQ and ZZF interpreted the data regarding the pulse-field gel electrophoresis; Axel provided suggestions on the conjugation assay; ZK and SH designed the work and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement

None declared

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Figures

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Figure 1

Dendrogram based on PFGE profiles of 6 KPC-2 and NDM co-producing *Escherichia coli* isolates. The dendrogram was produced by the UPGMA algorithm based on the Dice similarity coefficient included five PFGE groups as defined based on 85% similarity of PFGE profiles. CHβLs, carbapenem hydrolyzing β-lactamase.

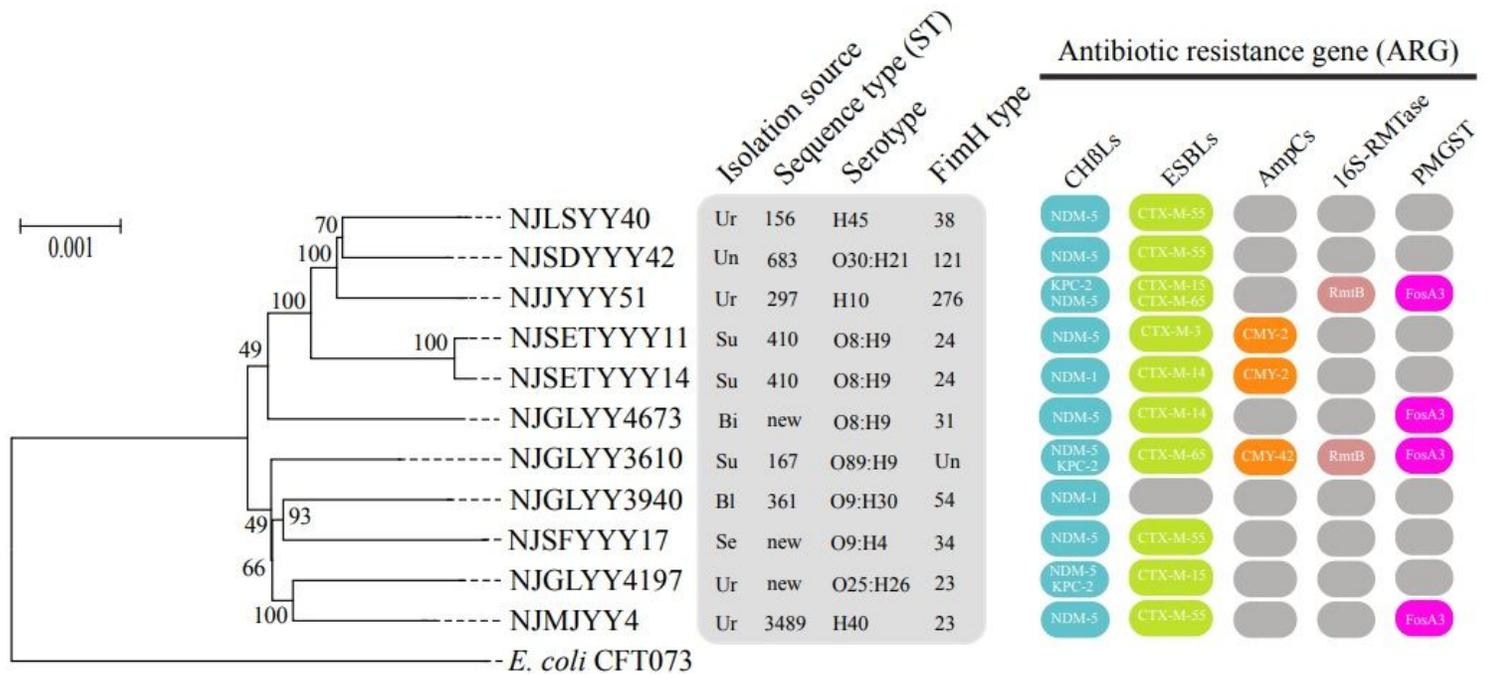


Figure 2

E. coli phylogenetic group tree-20200421-Li