Coexistence of $\text{bla}_{\text{NDM-5}}$ and $\text{tet}(X4)$ in international high-risk $\text{Escherichia coli}$ clone ST648 of human origin in China

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The emergence of pathogens is conferring resistance to last-resort therapies such as tigecycline, colistin, and carbapenems, limiting the therapeutic options, and raising concerns about the emergence of new “superbugs.” This study reports the first incident of a $\text{bla}_{\text{NDM-5}}$ and $\text{tet}(X4)$ co-harboring $\text{Escherichia coli}$ with resistance to carbapenem and tigecycline recovered as the causative agent of a urinary tract infection in a 94-year-old patient. The $\text{E. coli}$ strain ECCL209 carries multiple resistance genes [i.e., $\text{bla}_{\text{TEM-1B}}$, $\text{bla}_{\text{NDM-5}}$, $\text{bla}_{\text{CMY-2}}$, $\text{aadA22}$, florR, $\text{erm(B)}$, $\text{mph(A)}$, $\text{erm(42)}$, $\text{lnuG}$, $\text{qnrS1}$, and $\text{sul2}$] and exhibits resistance to almost all clinically used antibiotics. MLST analysis found that the strain belongs to ST648, considered a worldwide high-risk pandemic clone. Moreover, multiple plasmid incompatibility types were detected, i.e., $\text{IncHI1A}$, $\text{IncHI1B}$, $\text{IncFII}$, $\text{IncFIA}$, $\text{IncFIB}$, $\text{IncQ1}$, $\text{Col}$, and $\text{IncX4}$. Genetic analysis revealed that $\text{bla}_{\text{NDM-5}}$ and $\text{tet}(X4)$ genes were localized on two hybrid plasmids with multiple replicons. Continuous monitoring studies are suggested to quantify the antimicrobial resistance and assess the dissemination of such superbugs into a human healthcare setting.

KEYWORDS
antimicrobial resistance, $\text{Escherichia coli}$, $\text{bla}_{\text{NDM-5}}$, $\text{tet}(X4)$, coexistence, superbugs, hybrid plasmids

Introduction

Antimicrobial resistance (AMR) has been an emerging and increasing threat to global health (World Health Organization [WHO], 2014; Su et al., 2017). A report from 2016 predicted that global fatalities from infectious diseases caused by AMR will rise from 0.7 to 10 million by 2050, with a vast estimated inaction cost of US$100
trillion between 2016 and 2050 (O’Neill, 2016). The antibiotic-resistant bacteria (ARB) of particular interest are multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) (Basak et al., 2016). These ARBs are called superbugs, and they can cause severe bacterial infections due to their acquired and intrinsic resistance mechanisms and render the efficacy of many existing antibiotics (Potter et al., 2016; Acolatse et al., 2022).

Of particular concern is AMR among Gram-negative bacterial species, especially the carbapenem-resistant Escherichia coli (CRE), which is the leading cause of urinary tract infections (UTIs) and is challenging to treat with last-resort carbapenem antibiotics. Carbapenemases were developed to tackle bacteria producing extended-spectrum β-lactamases (ESBLs). However, Gram-negative bacteria have become resistant to this group of drugs by developing and/or acquiring bla genes encoding carbapenem hydrolyzing enzymes, named carbapenemases (Codjoe and Donkor, 2017; Nordmann and Poirel, 2019). Among the newly emerging carbapenemases, New Delhi Metallo-β-lactamase (NDM) is very important due to its widespread dissemination and allelic variations (Suay-Garcia and Pérez-Gracia, 2021). The pathogens harboring these genes resist almost all β-lactam antibiotics (Wu et al., 2019). Tigecycline and colistin were relatively effective and used as last-resort treatments to treat such infections caused by MDR and XDR bacteria (He et al., 2019). However, the recent discoveries of plasmid-mediated colistin resistance genes (mcr-1 to mcr-10) and/or the tigecycline resistance genes tet(X1) to tet(X15) among Enterobacteriaceae, especially in CRE, predict a return to the pre-antibiotic era and pose a severe threat to public health (He et al., 2019; Hussein et al., 2021). Furthermore, the recent discoveries of plasmid-mediated colistin resistance genes (mcr-1 to mcr-10) and/or the tigecycline resistance genes tet(X1) to tet(X15) among Enterobacteriaceae, especially in CRE, predict a return to the pre-antibiotic era and pose a severe threat to public health (He et al., 2019; Hussein et al., 2021).

To the best of our knowledge, herein, we identified the first case of XDR E. coli isolate co-harboring plasmid-mediated blanDM−5 and tet(X4) genes from a clinical sample of a human patient.

Materials and methods

Sample collection and identification

During a routine surveillance project on AMR, an E. coli isolate ECCL209 was recovered from a 94-year-old man, admitted for >6 months in the respiratory and critical care department at Shantou Hospital, Guangdong Province, China. The patient was diagnosed with UTI. The E. coli strain ECCL209 was identified by automated mass spectrometry systems (VitekMS, bioMerieux, Marcy l’Etoile, France) and further confirmed by PCR utilizing the primers specific to the uidA gene as reported previously (Shafiq et al., 2019).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was accomplished by Vitek 2 COMPACT (bioMerieux, Marcy l’Etoile, France) with AST-N334 cards for the following antimicrobial agents: amikacin (AMK), cefoperazone/sulbactam (SCF), ceftazidime (CTX), ceftepime (FEP), cefoxitin (FOX), peptidoglycan (PGR), ertapenem (ETP), imipenem (IMP), amoxicillin/clavulanic acid (AMC), cephalosporin (CXM), ceftriaxone (CRO), ceftazidime (CAZ), piperacillin/tazobactam (TZP), ticarcillin/clavulanic (TCC), ceftazidime-avibactam (CZA), ciprofloxacin (CIP), doxycycline (DOX), tigecycline (TIG), aztreonam (ATM), minocycline (MIC), tobramycin (TOB), trimethoprim/sulfamethoxazole (SXT), and colistin (COL). Antibiotic susceptibility for levofloxacin (LEV) was determined using Levofloxacin Susceptibility Test Paper (Thermo Scientific™ Oxoid™, Leicestershire, United Kingdom). Results for all antibiotics were interpreted following the standard of the Clinical and Laboratory Standard Institute (CLSI M100; 31st edition) guidelines, except imipenem, ertapenem, amoxicillin/clavulanic acid, and ceftazidime/avibactam for which the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were considered.

Detection of antibiotic resistance genes

Detection of common ESBL genes (i.e., blaTEM, blaCTX-M, and blashV), carbapenemases (blanDM, blaszPC, blarMP, blayVIM, and blazK), and tigecycline-resistant genes tet (X3 and X4) was performed using PCR to identify resistance genes. All the primers used in this study are summarized in Supplementary Table 1.

Mating assay

Conjugation experiments were performed according to a previously described method (Shafiq et al., 2019). The donor strain [blanDM and tet(X4)-positive E. coli] was diluted to the 0.5 McFarland standard and mixed with rifampicin-resistant recipient strain (E. coli C600) at a ratio of 1:1, respectively, on the microporous membrane. After cultures were incubated at 37°C for 12–14 h, the mixtures were collected and streaked on freshly
made Luria-Bertani (LB) agar plates containing tigecycline (2 mg/L), meropenem (2 mg/L), and rifampicin (300 mg/L). The presence of blaNDM and tet(X4) in transconjugants was confirmed by PCR and corresponding resistance phenotyping. The number of positive transconjugants per recipient calculated the transfer frequency of conjugation.

Whole-genome sequencing with Illumina and Nanopore

To determine the genomic background, the ECCL209 *E. coli* strain was subjected to whole-genome sequencing (WGS) on the Illumina Miseq and Oxford Nanopore Nanopore platforms. The total DNA of *E. coli* strain ECCL209 was collected from fresh overnight cultures using a DNA kit (QIAamp DNA Mini Kit, Germany) according to the manufacturer’s guidelines. The quality and quantity of extracted genomic DNA were measured and confirmed using a Nanodrop OD-1000 spectrophotometer (Thermo-Scientific®). DNA libraries were constructed using NEБNext UltraTM DNA Library Prep Kit for Illumina (NEB, USA) and sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). For the Nanopore platform, a Rapid Barcoding Sequencing Kit was used to construct the libraries and sequenced with a mini device (MinION), as previously reported (Maestri et al., 2019). Guppy base-calling software version 2.2 was used to generate fast5 files harboring the 1D DNA sequence from fast5 files. The quality of raw data from paired-end sequencing was checked using FastQC (version 0.11.6). Fastp (version 0.23.2) (Chen et al., 2018) was performed for the quality filtering to remove the low-quality reads, adapters, and poly-G tails. De novo assembly was accomplished using SPAdes (version 3.15.3) and Flye (version 2.8.3) with default parameters.

### TABLE 1 Genomic characteristics of *Escherichia coli* ST648 strain isolated from human origin.

| Characteristics of *E. coli* ST648 | Illumina (MiSeq) | Oxford Nanopore (MINION) |
|-----------------------------------|-----------------|--------------------------|
| Source                            | Human urine     | Human urine              |
| Genome size (bp)                  | 5,334,251       | 5,411,927                |
| Contigs                           | 176             | 4                        |
| G + C Content (%)                 | 50.2            | 50.3                     |
| tRNA                              | 83              | 88                       |
| rRNA                              | 5               | 22                       |
| No. of CDS                        | 5,303           | 5,423                    |
| Serotype                          | O83:H42         | O83:H42                  |
| ST 648                            | 648             | H58                      |
| ST                                | 648             | 648                      |
| Mobilome                          | IS5, ISL3, IS630, IS3, IS121, IS21, ISEpt1, IS4 | IS5, ISL3, IS6, IS91, ISEpt1, IS21, IS4, IS110, IS30, ISA1, IS630 |
| Virulome                          | iutA, terC, IpfA, SitA, yjfV, terC, hla, ciaA, ciaT, chuA, aii, iucC | traT, iucC, sitA, iutA, terC, IpfA, ciaA, yjfV, chuA, aii, aii, hlyE, hla |
| Resistome                         | [A0222]         | [A0222]                  |
| Aminoglycosides                   | bl__TEM--5, bl__TEM--1B, blCAT--2 | bl__TEM--5, bl__TEM--1B, blCAT--2 |
| β-lactams                         | floR            | floR                     |
| Chloramphenicol                   | flbR            | flbR                     |
| Macrolides                        | ermA, mphA, erm(42), laminA | ermA, mphA, erm(42), laminA |
| Quinolones                        | qnrS1           | qnrS1                    |
| Sulfonamides                      | sul2            | sul2                     |
| Tetracyclines                     | tet(X4), tetM   | tet(X4), tet(M)          |
| Plasmidome                        | IncH1A, IncH1B, IncFII, IncFIA, IncFIB, IncFIA, IncFIB, IncFII, IncFII, IncFII, IncQ1, Col, IncX4 | IncH1A, IncH1B, IncFII, IncFIA, IncFIB, IncFII, IncFII, IncFII, IncQ1, Col, IncX4 |
| BioProject accession number       | PRJNA850111     | PRJNA850111              |

ST, sequence type; CDS, coding sequences.

### Assembly annotation and genetic analysis of *Escherichia coli* ECCL209

The assembled genomes were subjected to determine the resistome, virulome, MLST, serotype, mobile genetic elements (MGEs), and plasmidome using online search tools such as ResFinder 4.0; VirulenceFinder 2.0, MLST 2.0, SerotypeFinder 2.0, MobileElementFinder, and PlasmidFinder 2.0, at the Center for Genomic Epidemiology (CGE).2 Genome annotation and visualization were performed using Prokka (version 1.14.6) and Proksee.3 Plasmid replicons were identified using Abricate (version 1.0.1)4 from the assemblies. EasyFig (version 2.2.2) was used to compare and visualize the region of interest between similar sequences. The sequence similarity search was performed using BLAST against the NCBI nucleotide database. The significant hits were investigated, and related information, including the source organisms and hosts, was visualized along the BLAST result tree using ggtree version 3.4.

### Results and discussion

The *E. coli* strain exhibited resistance against 19 antimicrobial agents, an XDR phenotype (Magiorakos et al., 2012), including SCF, FEP, FOX, CTX, ETP, IMP, AMC, LEV, TZP, CXM, CRO, CAZ, TCC, CZA, CIP, DOX, TIG, ATM, and MIC, while susceptible to AMK, TOB, SXT, and COL. The susceptibility data are shown in Table 2. The *E. coli* isolate ECCL209 was resistant to carbapenems.

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1. Shafiq et al. 10.3389/fmicb.2022.1031688
2. http://www.genomicepidemiology.org/services/, accessed on 30 June 2022.
3. https://proksee.ca/
4. https://github.com/tseemann/abricate
and tigecycline and harbored \textit{bla}_{\text{TEM}}, \textit{bla}_{\text{NDM}}, and \textit{tet}(X4) genes, amplified by PCR and subsequently confirmed by Sanger sequencing.

To determine the transmissibility of \textit{bla}_{\text{NDM}} and \textit{tet}(X4) genes, we performed conjugation experiments with a recipient \textit{E. coli} strain \textit{C600}. The outcomes of conjugation proved that the \textit{bla}_{\text{NDM}} and \textit{tet}(X4) genes in donor \textit{E. coli} isolate ECCL209, with their corresponding resistance against imipenem and tigecycline, were successfully moved to the recipient strain \textit{C600}, suggesting that \textit{bla}_{\text{NDM}} and \textit{tet}(X4) genes were located on conjugative plasmids. The cotransfer of \textit{bla}_{\text{NDM}} and \textit{tet}(X4) was at a frequency of \((1.67 \pm 0.2) \times 10^{-1}\) to \((3.12 \pm 0.1) \times 10^{-3}\) cells per recipient.

The main comprehensive results from the WGS analysis of Illumina and Nanopore are summarized in Table 1. The ECCL209 isolate was assigned as serotype O83:H42 using SerotypeFinder 2.0,\footnote{http://www.genomicepidemiology.org/services/, accessed on 30 July 2022.} which is an extraintestinal pathogenic \textit{E. coli} (ExPEC) primarily found in samples from animals, indicating their possible transmission from animal to humans (Abreu-Salinas et al., 2020; Shafiq et al., 2021a,b, 2022). MLST analysis revealed that \textit{E. coli} isolate ECCL209 in this study belonged to sequence type (ST648), which had been previously reported to carry \textit{bla}_{\text{CTX-M}}, \textit{bla}_{\text{CMY-2}}, \textit{bla}_{\text{NDM}}, \textit{bla}_{\text{OXA-48}}, and \textit{mcr-1} encoding genes and caused a significant proportion of infections in humans (Hornsey et al., 2011; Poirot et al., 2018; Chowdhury et al., 2022). This clonal lineage has emerged as a pandemic high-risk clone, being globally reported in humans, animals, and the environment (Hornsey et al., 2011; Fernandes et al., 2018; Furlan et al., 2020; Chowdhury et al., 2022; Landolsi et al., 2022). To the best of our knowledge, this is the first report of ST648, with \textit{bla}_{\text{NDM}} and \textit{tet}(X4).

Our resistome results confirmed aminoglycosides (\textit{aadA22}), amphenicols (\textit{floR}), \(\beta\)-lactams (\textit{bla}_{\text{TEM-1B}}, \textit{bla}_{\text{NDM-5}}, and \textit{bla}_{\text{CMY-2}}), sulfonamides (\textit{sul}2), macrolides [\textit{ermB}, \textit{mphA}, \textit{erm}(42)], quinolones (\textit{qnrS1}), and tetracycline-resistant genes [\textit{tet}(X4) and \textit{tetM}]. Moreover, we found chromosomal mutations in \textit{parE} (p. S458A), \textit{parC} (p. S801), and...
and gyrA (p. S83L, p. D87N), which encodes high-level resistance to fluoroquinolones (Mohsin et al., 2019). Multiple plasmids were detected in the E. coli ECCL209 strain, including, IncHI1A, IncHI1B, IncFII, IncFIA, IncFIB, IncQ1, Col, and IncX4. Detection of multiple plasmid types reflects the strains’ severity because all these replicons identified have the ability of horizontal transfer and play a vital role in spreading AMR genes (Rodríguez-Beltrán et al., 2021).

Regarding virulence genes, the presence of iutA (ferric aerobactin receptor), terC (tellurium ion resistance

![Figure 2](image1.png)

**Figure 2**
Structure of the blaNDM-5-carrying plasmid and comparison of the genetic context of blaNDM-5. (A) BLAST tree comparison of plasmid pECCL209-blaNDM5 with other homologous plasmids available in the NCBI database. (B) Structure of the blaNDM-5-carrying plasmid pECCL209. (C) Sequence comparison of the genetic context of a plasmid carrying blaNDM-5 gene from different sources. The arrows showed the direction of the transcription. Regions of >99% of homology are displayed by gray shading.

![Figure 3](image2.png)

**Figure 3**
Linear alignment of the selected blaNDM gene comparison with other homologous plasmids available in the NCBI database.
protein, IpfsA (long polar fimbriae), traT (outer membrane protein complements resistance), air (enteroaggregative immunoglobulin repeat protein), sitA (iron transport protein), hra (heat resistance agglutinin), yfcV (fimbrial protein), iusC (aerobactin synthetase), eilA (Salmonella Hia homolog), and chuA (outer membrane hemin receptor) were identified in *E. coli* strain ECCL209. These virulence genes could enhance bacterial pathogenicity, and a recent study also described their direct interaction with ARGs in terms of bacterial survivability, which need to be disclosed in future studies (Zhang et al., 2019).

To further understand the genetic contexts of *blaNDM-*5 and *tet(X4)*, we carried out long-read sequencing of *E. coli* ECCL209 isolate with the Oxford Nanopore MinION platform to obtain complete genome sequences. This assembled genome had four contigs, with a total length of 5,411,927 bp and an average G + C content of 50.31%. Bioinformatic analysis revealed that isolate ECCL209 harbored a chromosome and three circular plasmids comprising pECCL209-*tet(X4)-190-kb*, pECCL209-*blaNDM5-157-kb*, and pECCL209-*blaCMY-2-36-kb*.

pECCL209-*tet(X4)* was a 190,682-bp plasmid co-fused with IncH1A, IncH1B, and IncFII, forming multiple replicon plasmids. Similarly, a fusion plasmid has been previously reported from China recently, where a tet(X4) gene was located in *Enterobacter cloacae* on a hybrid plasmid (~190 kb) with IncFIA, IncH1A, and IncH1B replicons (Wu et al., 2022). This high homology of plasmids from animal and human origin suggests that *tet(X4)*-carrying plasmids could be conjugated from *E. cloacae* to *E. coli*. The BLASTn search was performed against the NCBI database to examine the sequence similarity of pECCL209-*tet(X4)-190-kb* and pECCL209-*blaNDM5-157-kb*. Phylogenetic analysis revealed that the pECCL209-*tet(X4)* plasmid was similar to other bacterial strains with ≥90% query coverage. Most of the plasmid sequences matched with pECCL209-*tet(X4)* were from animal origins, while this is the first human-origin *E. coli* plasmid harboring tet(X4) resistant gene (*Figure 1A*). The result showed that the pECCL209-*tet(X4)*-like plasmid might have been widely spread in different species of *Enterobacteriaceae*. pECCL209-*tet(X4)-190-kb* displayed a mosaic structure harboring five AMR genes, including *flor* (phenicol resistance), *qnrS* (quinolone resistance), *erm*(B) (*blaNDM-*5), *aph* (aaminoglycosides resistance), and *traT* (>99% identity) with other *blaNDM-*5-carrying plasmids in *K. pneumoniae* plasmid pEH13_2 (GeneBank accession no. CP089099.1) and *E. coli* plasmid pYSP8-1-CTX-M-14 (GeneBank accession no. CP037912.1) of human and animal origin, respectively, suggesting that *blaNDM-*5-carrying plasmids had widely disseminated in China (*Figure 2A*). The *blaNDM-*5 gene resided in a complex region of the plasmid pECCL209-*blaNDM5-157,741-bp*. The plasmid carried other resistance genes, including *aadA*2 (aminoglycosides resistance), *erm*(B), *erm*(D), *mph*(A) (macrolides resistance), *tet*(M) (tetracycline resistance), and *sul2* (sulfonamide resistance), and MGEs found in the MDR region, including *IS26*, *ISVsa3*, *IS5*, *ISEc9*, *ISKox3*, and *IS91* (*Figure 2B*). The *blaNDM-*5 gene was located within a 10.8-kb region, which was highly similar (99% identity) to *E. coli* plasmid pGZ3_NDM5 (GeneBank accession no. CP017981.1) obtained from patient urine with intra-abdominal infections and *Salmonella enterica* plasmid unnamed2 (GeneBank accession no. CP019444.1) collected from a patient stool in China. In the plasmid backbone of pECCL209-*blaNDM5-10kb*, *IS5* was inserted with *ISAba125* upstream of *blaNDM-*5, and the *ble* (*blaNDM*-*Aba125*) and *dsbD* (*blaNDM*-*ABa125*) were located downstream from *blaNDM-*5 as shown *Figure 2C*. Interestingly, on sequence alignment of our plasmid pECCL209-*blaNDM5* with other identical plasmids ≥60% BLAST query coverage found that the *blaNDM-*5 region in other strains was mostly missing as shown in *Figure 3*, suggesting that *blaNDM-*5 in pECCL209-*blaNDM5* was captured from other mobile elements.

**Conclusion**

As far as we know, this is the first report that emphasized the emergence of high-risk *E. coli* clone ST648 of a human origin, which carries the mobile carbapenem and tigecycline resistance determinants *blaNDM-*5 and *tet(X4)*, respectively. Regardless of their low prevalence rate in humans and animal-associated sources, the mobile plasmid-mediated resistance genes in such superbugs can pose a significant threat to public health. Therefore, continuous monitoring of such MDR and XDR bacteria in humans, animals, and the environment should be considered under the aegis of the One Health approach and to guide the deployment of public health interventions before clinical cases increase.
Data availability statement

The sequence data mentioned in this present study were deposited to the GenBank NCBI database under the BioProject PRJNA850111 with accession number: SRR19844396.

Ethics statement

Ethical approval was provided by the Human Research Ethics Committee of Shantou Central Hospital and Shantou University Medical College (Ref 047 and SUMC-2021-51, respectively). Consent forms from the patients were waived by the Ethical Committee as all the clinical samples were obtained from the hospital laboratory.

Author contributions

MS and XJ designed the experiments. MS, MZ, and XL performed the experiments. MS wrote the original manuscript. BP and YY helped in the analysis. JH, HB, FY, and AA reviewed the experiments. MS wrote the original manuscript. MS and XJ designed the experiments. MS, MZ, and XL performed the experiments. MS wrote the original manuscript. BP and YY helped in the analysis. JH, HB, FY, and AA reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1031688/full#supplementary-material

Supplementary table 1
List of primers used in this study.

Supplementary table 2
Antibiotic susceptibility profile of Escherichia coli strain ECCL209.

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