Computational and Structural Biotechnology Journal 21 (2023) 847–855

Contents lists available at ScienceDirect

Computational and Structural Biotechnology Journal

journal homepage: www.elsevier.com/locate/csbj

Molecular epidemiology and genomic insights into the transmission of carbapenem-resistant NDM-producing *Escherichia coli*

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ARTICLE INFO

Article history:
Received 1 November 2022
Received in revised form 5 January 2023
Accepted 7 January 2023
Available online 9 January 2023

Keywords:
Escherichia coli
Carbapenem-resistant
NDM
Plasmid
Whole-genome sequencing

ABSTRACT

Escherichia coli is a leading cause of nosocomial infections. Carbapenem-resistant *E. coli* (CREC), which has been frequently isolated in recent years because of the widespread use of carbapenems, poses a significant challenge to clinical anti-infection treatment. In this study, a total of 27 CREC strains were identified from a set of 795 *E. coli* isolates collected over a two-year period from a tertiary hospital in China. Whole-genome sequencing revealed that 17 strains carried the bla_{NDM-5} gene, 5 strains carried the bla_{NDM-1} gene, 1 strain carried the bla_{NDM-7} gene, and the remaining 4 strains carried the bla_{KPC-2} gene. All 23 NDM-producing *E. coli* strains were resistant to all antibiotics except tigecycline, colistin, and cefiderocol. Nine different sequence types (STs) were identified, with ST410 and ST167 being the most prevalent. All of the bla_{NDM} genes were located on conjugatable plasmids. We identified five different plasmid replicon types ranging in size from 20 kb to 200 kb, with the IncX3-type plasmid, 46 kb in size, being a key factor in facilitating the horizontal transmission of the bla_{NDM} gene in *E. coli*. The structure surrounding the bla_{NDM} gene was relatively conserved and mainly contained the following structures: IS*3000*-IS*Aba125*-IS*5*-bla_{NDM}-ble_{MBL}-*trpF*-*dsbC*-IS*26*. However, the plasmid backbone structure was highly variable, which indicates that the bla_{NDM} gene has already spread horizontally among different types of plasmids. In addition, we discovered two copies of the bla_{NDM-5} gene in a single plasmid (pEC29-NDM-5), with an identical structure around the gene and the complete sequence of the class 1 integron. Our findings detail the prevalence of CREC in a tertiary hospital in China, and the emergence of multiple copies of the bla_{NDM-5} gene on a single plasmid needs our attention.

1. Introduction

Escherichia coli, a member of the Enterobacterales family, is one of the most common conditional pathogens. It carries a variety of virulence factors, such as fimbriae, capsules, toxins, and lipopolysaccharide. Numerous infections, including acute gastroenteritis, urinary tract infections, abdominal infections, and bloodstream infections, can be caused by *E. coli*. Antibiotics such as β-lactams are used as part of the standard treatment for such infections. One of the important antibacterial agents used in the clinical treatment of severe bacterial infections is carbapenems, a class of β-lactam antibiotics with strong antibacterial activity, a broad antibacterial spectrum, and low toxicity. Unfortunately, the extensive use of these medicines has led to an increase in antibiotic resistance.

The main mechanism by which Enterobacterales achieve carbapenem resistance is through the production of carbapenemases [1–3]. New Delhi metallo-β-lactamase (NDM) has been the most commonly detected carbapenemase in *E. coli* [4,5]. NDM is an Ambler class B β-lactamase, which is the most common type of carbapenemase, and NDM confers resistance to nearly all β-lactams except aztreonam [3,6]. Isolates producing the NDM enzyme have been detected worldwide. The NDM producing *E. coli* strains frequently carrying extended spectrum lactamases (ESBLs), which are also resistant to aztreonam [7]. Furthermore, *E. coli* isolates capable of producing carbapenemases frequently showed higher rates of resistance to other antibiotics, such as aminoglycosides and quinolones, which severely limits clinical treatment options [8].

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https://doi.org/10.1016/j.csbj.2023.01.004

Although there have been reports of carbapenem-resistant Enterobacterales (CRE) strains worldwide, most research on the topic focuses on carbapenem-resistant *Klebsiella pneumoniae* [9–14]. In contrast, carbapenem-resistant *E. coli* is isolated less frequently in the clinical settings, and the strains isolated in different regions differ in terms of resistance phenotypes and clonal distribution to varying degrees [15]. To better understand the molecular epidemiological traits and transmission dynamics of carbapenem-resistant *E. coli* in a tertiary hospital in China, 795 *E. coli* strains were collected between January 2016 and December 2017. The carbapenem-resistant strains were identified by antimicrobial susceptibility testing and then subjected to whole-genome sequencing. The antimicrobial resistance genes, epidemiological characteristics, and transmission dynamics of strains with NDM-carrying plasmids were further investigated.

2. Materials and methods

2.1. Bacterial isolation

Between January 2016 and December 2017 in a tertiary hospital in China, 795 *E. coli* strains were isolated from various types of samples. Duplicate strains were those that came from different samples on the same patient or samples taken from the same patient at various times; only the first strain was utilized for further study. All of the isolates were identified using the BD Bruker MALDI-Type system and then tested for antimicrobial susceptibility using the BD Phoenix system for primary screening.

2.2. Antimicrobial susceptibility testing for carbapenem-resistant strains

Carbapenem-resistant strains collected after primary screening were further subjected to antimicrobial susceptibility testing using standard broth microdilution tests following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The following antimicrobial agents: ceftazidime, cefotaxime, cefepime, cefoxitin, aztreonam, fosfomycin, imipenem, meropenem, amikacin, ciprofloxacin, levofloxacin, gentamicin, sulfamethoxazole/trimethoprim, colistin, tigecycline and cefiderocol were used for testing. Antimicrobial susceptibility was determined using breakpoints approved by the CLSI [16]. For tigecycline minimum inhibitory concentration (MIC) detection, standard broth microdilution tests were adopted using fresh (< 12 h) Mueller-Hinton broth (Cation-adjusted, Oxoid LTD, Basingstoke, Hampshire, England). Cefiderocol MICs were determined in iron-depleted cation-adjusted Mueller-Hinton broth. *E. coli* ATCC 25922 was used for quality control. As there are no CLSI breakpoints for tigecycline, the FDA standard was adopted (https://www.fda.gov/drugs/development-resources/tigecycline-injection-products). The interpretation of the colistin MIC followed the EUCAST guidelines (Breakpoints for 2021, https://eucast.org/).

2.3. Whole-genome sequencing

Isolates confirmed to be resistant to carbapenems were sent for whole-genome sequencing using the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) and a long-read MinION sequencer (Nanopore, Oxford, UK). Unicycler (v0.4.7) was used in conservative mode to assemble both short Illumina reads and long MinION reads in a hybrid manner. Pilon was used to create complete circular contigs that were then repeatedly corrected using Illumina reads until no change was found [17]. The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) server automatically generated annotations for the entire genome sequence.

2.4. Genomic characterizations and phylogenetic analysis of bla_{NDM}-positive strains

The BacWGSTdb 2.0 server was used to analyse the multilocus sequence typing (MLST), acquired antibiotic resistance genes, and plasmid replicons of the bla_{NDM}-positive strains [18–20]. The phylogenetic relationship between bla_{NDM}-positive strains was initially analysed using the BacWGSTdb server with core genome MLST (cgMLST) approaches. The phylogenetic tree was built using the neighbor joining (NJ)/unweighted pair group method with the arithmetic mean (UPGMA) phylogeny method (MAFFT version 7) [21], which is based on a core genome single nucleotide polymorphism (SNP) strategy. The maximum parsimony algorithm was used to create a phylogenetic tree from the resulting SNPs after recombination regions were removed [22].

2.5. PFGE analysis

Genomic DNA was digested with the restriction enzyme *Xba*I (TaKaRa, Dalian, China), and DNA fragments were then separated by electrophoresis in 1% agarose III (Sangon, Shanghai, China) with 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA; pH 8.0) buffer using a CHEF apparatus (CHEF Mapper XA, Bio-Rad, USA) at 14 °C and 6 V/cm and with alternate pulses at a 120° angle in a 5- to 35-s pulse time gradient for 22 h. BioNumerics 7.0 (Applied Maths, Austin, TX, USA) software was used to analyse the PFGE results.

2.6. Conjugation experiment and S1-PFGE

bla_{NDM}-positive *E. coli* strains were used as donors, and the sodium azide-resistant *E. coli* strain J53 was used as the recipient. Transconjugants were selected on MH agar plates supplemented with imipenem (4 mg/L) and sodium azide (150 mg/L). *E. coli* J53 transconjugants were identified using the VITEK MS system, and the bla_{NDM} gene was further confirmed by PCR and Sanger sequencing. The conjugation efficiency was measured and calculated following the protocol in https://openwetware.org/wiki/conjugation. S1-PFGE was conducted following the protocol of Barton et al. [23].

2.7. Characterization of the NDM-bearing plasmid and the genetic background of bla_{NDM}

Circular comparisons of the bla_{NDM}-carrying plasmid were performed using BLAST Ring Image Generator (BRIG) based on concentric rings [24]. By using ISfinder, the insertion elements (ISs) found on the plasmids were predicted [25]. The comparison of the genetic location and background of bla_{NDM} between different plasmids was performed using EasyFig 2.2.3 [26].

2.8. Nucleotide sequence accession numbers

The genome sequences of the bla_{NDM}-carrying *E. coli* isolates were deposited in the NCBI GenBank database under the BioProject accession number PRJNA608094.

3. Results

3.1. Strains and clinical metadata

In total, 795 *E. coli* clinical strains were collected during the study period. Of the patients, 39.5 % (314/795) were male and 60.5 % (481/795) were female. Children made up 1.8 % of the population (14/795), while adults, who were mostly middle-aged and elderly patients over the age of 45 years, made up 98.2 % of the population (781/795). Most of the specimens (541/795, 67.9 %) were urine specimens, followed by sputum (88/795, 11.0 %), pus (58/795, 7.2 %),

Table 1
Susceptibility of the 795 *Escherichia coli* clinical strains to 16 antimicrobial agents.

Antimicrobial agent	Susceptible		Intermediate		Resistant	
	number	(%)	number	(%)	number	(%)
piperacillin	147	18.5	11	1.4	637	80.1
sulfamethoxazole/ trimethoprim	363	45.7	1	0.1	431	54.2
cefotaxime	383	48.2	4	0.5	408	51.3
ciprofloxacin	418	52.6	24	3.0	353	44.4
cefepime	411	51.7	40	5.0	344	43.3
levofloxacin	435	54.7	20	2.5	340	42.8
gentamicin	458	57.6	3	0.4	334	42.0
aztreonam	511	64.3	54	6.8	230	28.9
ampicillin/ sulbactam	351	44.1	238	29.9	206	26.0
chloramphenicol	567	71.3	42	5.3	186	23.4
ceftazidime	576	72.5	61	7.7	158	19.8
amoxicillin/ clavulanate	581	73.1	132	16.6	82	10.3
piperacillin/ tazobactam	718	90.3	13	1.6	64	8.1
amikacin	754	94.8	4	0.5	37	4.7
imipenem	766	96.4	2	0.2	27	3.4
meropenem	766	96.4	2	0.2	27	3.4

blood (46/795, 5.7 %), wound secretion (24/795, 3.0 %), ascites, bile, and cerebrospinal fluid specimens. The main sample collection departments were as follows: rehabilitation (284/795, 35.7 %), urology (162/795, 20.3 %), gynecology (62/795, 7.7 %), neurology (35/795, 4.3 %), neurosurgery (32/795, 4.0 %), oncology (31/795, 3.9 %), and the intensive care unit (22/795, 2.8 %).

Table 1 shows the resistance rates of the 795 *E. coli* isolates to the 16 antimicrobial agents. These strains were most resistant to piperacillin (637/795, 80.1 %), followed by sulfamethoxazole/trimethoprim (431/795, 54.2 %) and cefotaxime (408/795, 51.3 %); however, the strains were susceptible to imipenem, meropenem, and amikacin.

3.2. Clinical data of carbapenem-resistant E. coli strains

From the initial antimicrobial susceptibility testing, 27 carbapenem-resistant *E. coli* strains were identified. According to whole-genome sequencing, four strains carried bla_{KPC-2}, five strains carried bla_{NDM-1}, seventeen strains carried bla_{NDM-5}, and one strain carried bla_{NDM-7}. The predominant carbapenemase gene present in these strains was bla_{NDM}, with the bla_{NDM-5} subtype being the majority. Table 2 displays the clinical data about these 23 bla_{NDM}-positive *E. coli* strains. The specimens came from urine (17 cases), sputum (3 cases), wounds (2 cases), and pharyngeal swabs (1 case). Twelve men and eleven women, with a mean age of 55.5 ± 19.4 years, made up the patient population. The oldest patient was 89 years old, and the youngest was 21. According to the distribution by ward, there were 15 strains (65.3 %) from the department of rehabilitation, four strains (17.4 %) from the department of neurosurgery, two strains (8.7 %) from the intensive care unit, one strain (4.3 %) from the department of oncology, and one strain (4.3 %) from the department of internal medicine. Most of these patients were initially admitted to the hospital for a variety of underlying diseases, and information from their medical records revealed that most of them had histories of multiple hospitalizations, transfers, invasive operations, and the administration of various antimicrobial agents while they were in the hospital.

The results of the antimicrobial susceptibility testing of bla_{NDM}-positive strains are presented in Table S1. All strains were multidrug-resistant, with 100% resistance to ceftazidime, cefotaxime, cefepime, cefoxitin, aztreonam, imipenem, meropenem, ciprofloxacin, levofloxacin, and sulfamethoxazole/trimethoprim; 95.6 % resistance to fosfomycin and gentamicin; and 82.6 % resistance to amikacin; however, the strains were completely sensitive to tigecycline, colistin, and cefiderocol.

3.3. PFGE analysis of bla_{NDM}-positive strains

Utilizing the BioNumerics program, the PFGE results of the bla_{NDM}-positive strains were analysed [27]. Three clonal groups with high homology (number of band differences < 3) were found and designated the type I, type II, and type III clonal groups (Fig. 1). The type I clonal group strains EC12, EC15, and EC18 were found in the neurosurgery department and the intensive care unit (ICU); the type II clonal group strains EC19, EC20, EC24, EC25, and EC27 were found in the rehabilitation department, the internal medicine department, and the ICU; and the type III clonal group strains EC9 and EC10 were found in the rehabilitation department. The relationships between the other strains were more distant.

3.4. Genomic characterization of bla_{NDM}-positive strains

Illumina and Nanopore sequencing were used to obtain the complete genome sequences of the 23 bla_{NDM}-positive strains. Table S2 provides a summary of the genomic characterization of these strains. These strains' chromosomes ranged in size from 4.6 Mbp to 5.0 Mbp, contained 4600–5200 CDS, and had a GC content of approximately 50 %. A total of nine different ST types were identified, including ST410 (EC27, EC25, EC19, EC20, EC24), ST167 (EC11, EC31, EC22, EC29), ST6388 (EC12, EC15, EC18), ST744 (EC3, EC13, EC7), ST224 (EC9, EC10), ST10 (EC26, EC4), ST359 (EC21, EC14), ST1193 (EC8), and ST361 (EC5). The most prevalent ST type among them was ST410, followed by ST167.

3.5. Phylogenetic analysis of bla_{NDM}-positive strains

The phylogenetic tree created using the cgMLST strategy is displayed in Fig. 2. These strains were grouped into clusters, and strains from the same cluster were further separated into branches, each with a different number of allelic differences between strains. There was only one allele that separated the three ST410 strains (EC19, EC20, and EC24), one separated the three ST6388 strains (EC12, EC15, and EC18), and the two ST224 strains (EC9, EC10) had the same alleles.

In Fig. 3, a phylogenetic tree was created using the core genome single nucleotide polymorphism (cgSNP) strategy, and the 23 bla_{NDM}-positive strains could be divided into several evolutionary branches. We discovered that the MLST and cgSNP typing results were highly congruent, and the cgSNP phylogenetic tree identified strains with the same sequence type that were topologically aggregated. These strains carried multiple ARGs, including the β-lactam resistance genes bla_{CMY-42}, bla_{CTX-M}, bla_{NDM-1}, bla_{NDM-5}, bla_{NDM-7}, bla_{OXA-1}, bla_{SHV-12} and bla_{TEM-1B}; the aminoglycoside resistance genes *aac(3)-lld*, *aac(3)-iva*, *aac(6′)-lb-cr*, *aadA*, *aph(3″)-lb*, *aph(3′)-la*, *aph(6)-ld*, *armA* and *rmtB*; the fluoroquinolone resistance genes *qnrS1*, *oqxA* and *oqxB*; the chloramphenicol resistance genes *catA*, *catB* and *floR*; the tetracycline resistance genes *tet*(A) and *tet*(B); the trimethoprim resistance gene *dfrA17*; the sulfonamide resistance genes *sul1* and *sul2*; the fosfomycin resistance gene *fosA3*; and the macrolide resistance gene *mph*(A).

3.6. Characterization of the bla_{NDM}-bearing plasmid

In accordance with the findings of the S1-PFGE (Fig. S1), whole-genome sequencing revealed that the bla_{NDM} gene was located on plasmids in all 23 bla_{NDM}-positive *E. coli* strains, with plasmid sizes ranging from 24,665 to 168,756 bp (Table 3). The IncX3 (n = 19), IncFIA (n = 1), IncX3/IncFIB (n = 1), IncL/M (n = 1), and IncA/C2 (n = 1) plasmid replicons were among the five plasmid replicons in

Table 2
Clinical metadata of the 23 bla_{NDM}-carrying *E. coli* strains.

Strain	NDM sub-type	Patient gender	Patient age	Department	Clinical diagnosis	Separation time	Specimen type
EC3	bla_{NDM-5}	male	60	rehabilitation	intracranial injury	2016–01–22	sputum
EC4	bla_{NDM-5}	male	42	neurosurgery	intracranial injury	2016–02–26	urine
EC5	bla_{NDM-5}	male	35	rehabilitation	intracranial injury	2016–03–18	wound
EC7	bla_{NDM-5}	female	73	rehabilitation	spinal membrane tumor	2016–04–26	urine
EC8	bla_{NDM-1}	female	61	rehabilitation	multiple injuries	2016–07–18	urine
EC9	bla_{NDM-1}	female	59	rehabilitation	meningioma	2016–07–28	urine
EC10	bla_{NDM-1}	female	70	rehabilitation	cerebral hemorrhage	2016–07–25	urine
EC11	bla_{NDM-1}	female	75	rehabilitation	intracranial hemorrhage	2016–07–24	urine
EC12	bla_{NDM-5}	male	56	neurosurgery	cerebral hemorrhage	2016–08–09	urine
EC13	bla_{NDM-5}	male	43	rehabilitation	post-operative aortic coarctation	2016–09–28	urine
EC14	bla_{NDM-1}	female	25	rehabilitation	post-traumatic brain injury	2016–11–16	wound
EC15	bla_{NDM-5}	male	21	ICU	multiple injuries	2016–12–01	urine
EC18	bla_{NDM-5}	male	44	neurosurgery	cranial injury	2016–12–20	urine
EC19	bla_{NDM-5}	female	80	rehabilitation	cerebral infarction	2017–05–28	urine
EC20	bla_{NDM-5}	female	67	rehabilitation	carbon monoxide poisoning delayed encephalopathy	2017–06–22	pharyngeal swabs
EC21	bla_{NDM-5}	female	33	rehabilitation	intracranial injury	2017–07–07	urine
EC22	bla_{NDM-5}	male	89	oncology	pulmonary malignancy	2017–07–11	sputum
EC24	bla_{NDM-5}	male	68	rehabilitation	intracranial hemorrhage	2017–08–17	urine
EC25	bla_{NDM-7}	male	57	internal medicine	pulmonary Infection	2017–08–17	urine
EC26	bla_{NDM-5}	female	58	neurosurgery	post-traumatic brain injury syndrome	2017–09–06	urine
EC27	bla_{NDM-5}	female	58	ICU	cardiac arrest	2017–10–16	urine
EC29	bla_{NDM-5}	male	22	rehabilitation	intracranial injury	2017–11–21	urine
EC31	bla_{NDM-5}	male	81	rehabilitation	cerebral infarction	2017–12–06	sputum

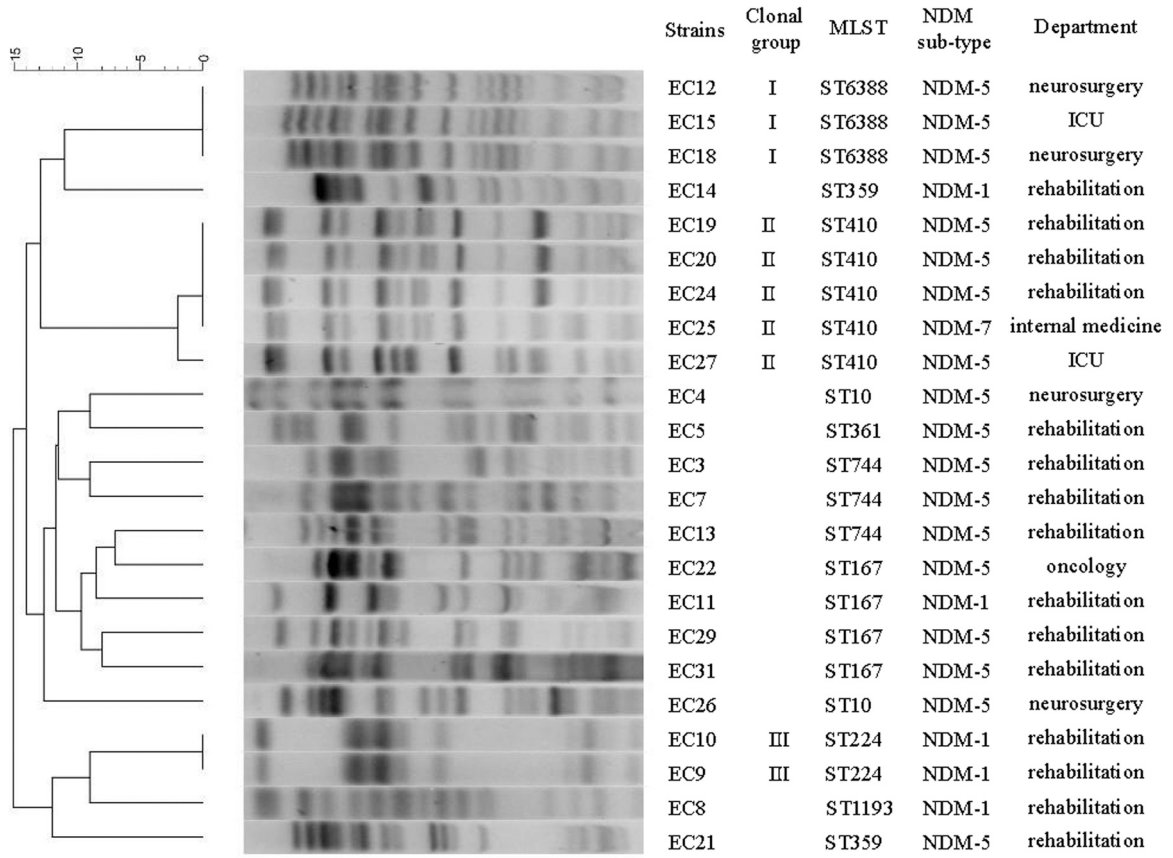

Fig. 1. PFGE results of 23 bla_{NDM}-positive *E. coli* strains analysed by the BioNumerics program. Three clonal groups were identified and categorized as type I, type II, and type III clonal groups based on their high homology (number of band differences < 3).

plasmids carrying the bla_{NDM} gene. The IncX3-type, with plasmid sizes ranging from 24,665 to 61,845 bp, was the most prevalent plasmid replicon harboring the bla_{NDM} gene. Additionally, we discovered instances when a single plasmid included two plasmid replicons (e.g., EC22). All plasmids carrying the bla_{NDM} gene could be successfully conjugated into *E. coli* J53, and the conjugation efficiency ranged from 3.99×10^{-5} to 8.81×10^{-8} (Table 3). Overall, IncX3-type plasmids had a higher conjugation efficiency than IncL/M-, IncA/C2-, and IncFIA-type plasmids, indicating that they can be horizontally transferred more easily. The MICs of 23 *E. coli* J53 transconjugants of bla_{NDM} to 15 antimicrobial agents are presented in Table S3. The strains were all resistant to carbapenems, and some

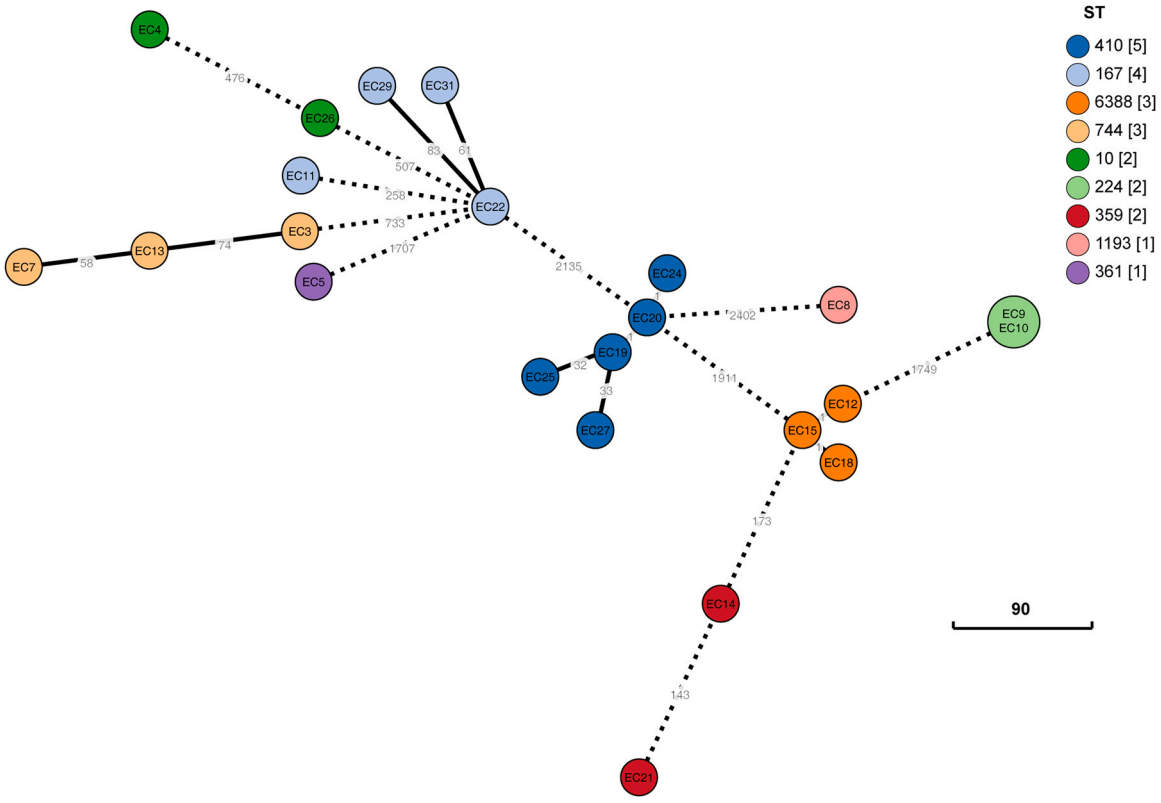

Fig. 2. Phylogenetic tree of 23 *bla*$_{NDM}$-positive *E. coli* strains created using the cgMLST strategy. The distance between each circle depicts clonal relationships between different isolates. Each sequence type is indicated by the color of the circle. The numbers enclosed in square brackets denote the number of isolates from each sequence type.

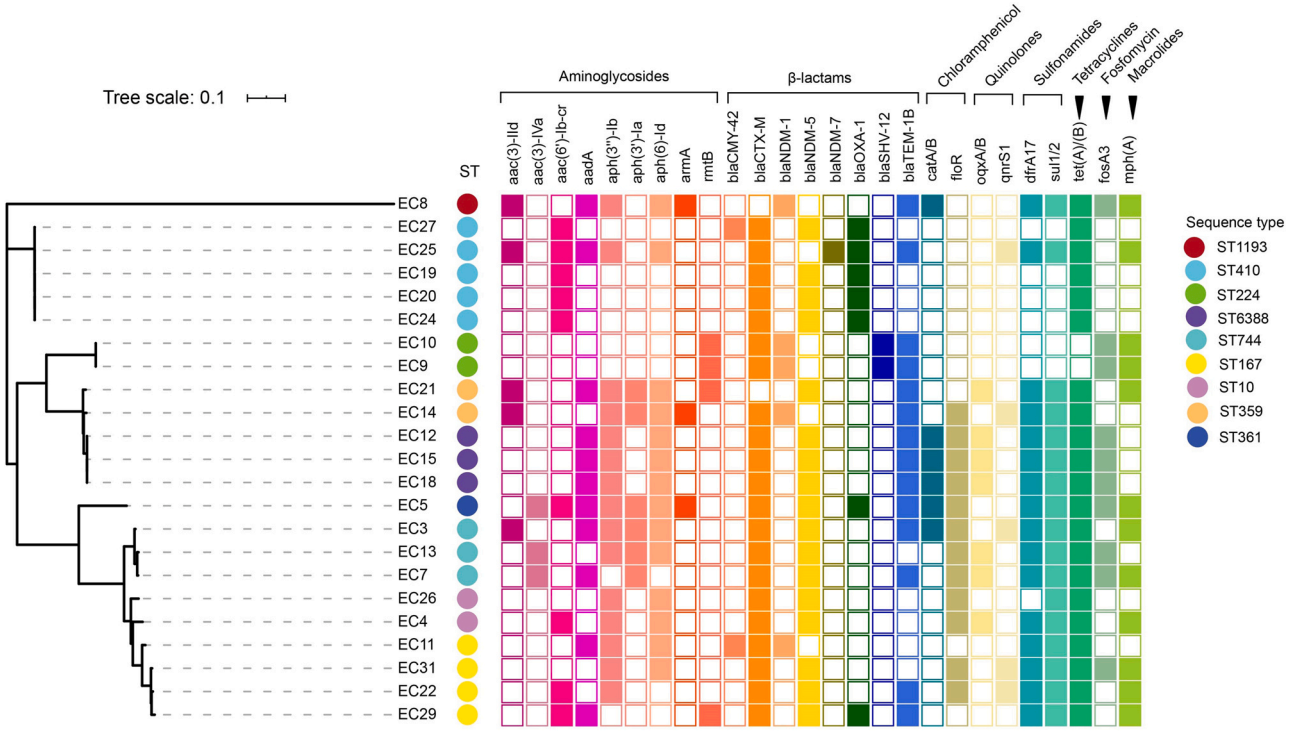

Fig. 3. Phylogenetic tree created using the cgSNP strategy. Antimicrobial resistance gene and sequence type analyses were performed using WGS data from 23 *bla*$_{NDM}$-positive bacteria. The antimicrobial resistance gene is presented in cells by various colors; however, the gene is absent in cells that are blank. Each circle color designates a specific sequence type.

Table 3
Genomic characterizations of bla_{NDM}-carrying plasmids and their conjugation efficiency.

Strains	NDM sub-type	Size (bp)	GC %	Plasmid replicons	Conjugation efficiency
EC18	bla_{NDM-5}	24,665	49.1 %	IncX3	5.43×10^{-6}
EC12	bla_{NDM-5}	24,944	49.1 %	IncX3	1.63×10^{-6}
EC15	bla_{NDM-5}	24,944	49.1 %	IncX3	4.00×10^{-7}
EC19	bla_{NDM-5}	46,161	46.7 %	IncX3	1.45×10^{-7}
EC20	bla_{NDM-5}	46,161	46.7 %	IncX3	3.57×10^{-7}
EC21	bla_{NDM-5}	46,161	46.7 %	IncX3	2.92×10^{-6}
EC24	bla_{NDM-5}	46,161	46.7 %	IncX3	1.05×10^{-6}
EC25	bla_{NDM-7}	46,161	46.6 %	IncX3	3.99×10^{-5}
EC26	bla_{NDM-5}	46,161	46.7 %	IncX3	3.65×10^{-7}
EC27	bla_{NDM-5}	46,161	46.7 %	IncX3	5.38×10^{-5}
EC5	bla_{NDM-5}	46,161	46.7 %	IncX3	8.14×10^{-6}
EC7	bla_{NDM-5}	46,161	46.7 %	IncX3	4.62×10^{-6}
EC4	bla_{NDM-5}	46,164	46.7 %	IncX3	1.14×10^{-7}
EC13	bla_{NDM-5}	47,499	46.6 %	IncX3	1.27×10^{-6}
EC3	bla_{NDM-5}	48,290	47.0 %	IncX3	7.66×10^{-7}
EC31	bla_{NDM-5}	48,521	47.0 %	IncX3	4.17×10^{-7}
EC11	bla_{NDM-1}	49,849	48.6 %	IncX3	1.51×10^{-6}
EC10	bla_{NDM-1}	61,845	49.0 %	IncX3	2.12×10^{-6}
EC9	bla_{NDM-1}	61,845	49.0 %	IncX3	4.50×10^{-5}
EC14	bla_{NDM-1}	118,444	51.3 %	IncL/M	8.81×10^{-8}
EC29	bla_{NDM-5}	147,459	53.1 %	IncFIA	3.68×10^{-8}
EC22	bla_{NDM-5}	168,221	50.1 %	IncX3/IncFIB	4.35×10^{-7}
EC8	bla_{NDM-1}	168,756	51.1 %	IncA/C2	6.47×10^{-8}

transconjugants also developed resistance to other antimicrobial agents (e.g., amikacin and fosfomycin). This indicates that the plasmid can also carry additional ARGs or that more resistance plasmids entered the transconjugants during the conjugation.

3.7. Genetic background of bla_{NDM}

Plasmids carrying the bla_{NDM} gene contain additional elements, including antibiotic resistance genes, mobile elements, conjugation-associated elements, and plasmid stability-associated genes. Table 3 shows that the GC content of the bla_{NDM}-carrying plasmids with different plasmid replicons. The IncX3 plasmid had the lowest GC content (46–49 %) compared to the IncFIA, IncL/M, and IncA/C2 plasmids, while IncFIA was the plasmid replicon type with the highest GC content (53.1 %). In addition, IncX3-type plasmids were found to carry the following subtypes of bla_{NDM} in this study: bla_{NDM-1}, bla_{NDM-5} and bla_{NDM-7}. Fig. 4 compares the sequences of IncX3 plasmids of various sizes, and the IncX3 plasmid backbone structure is highly conserved and carries only the bla_{NDM} gene for antibiotic resistance (except for a few plasmids that also carry bla_{SHV-12}). Fig. 5 compares the sequences of the plasmids IncA/C2, IncL/M, IncX3/IncFIB, and IncFIA. These plasmids are significantly larger (> 100 kb) than IncX3 plasmids and there are very few structural similarities between them other than the antibiotic resistance gene region. In addition to bla_{NDM}, these large plasmids also contain a wide range of other antibiotic resistance genes, including

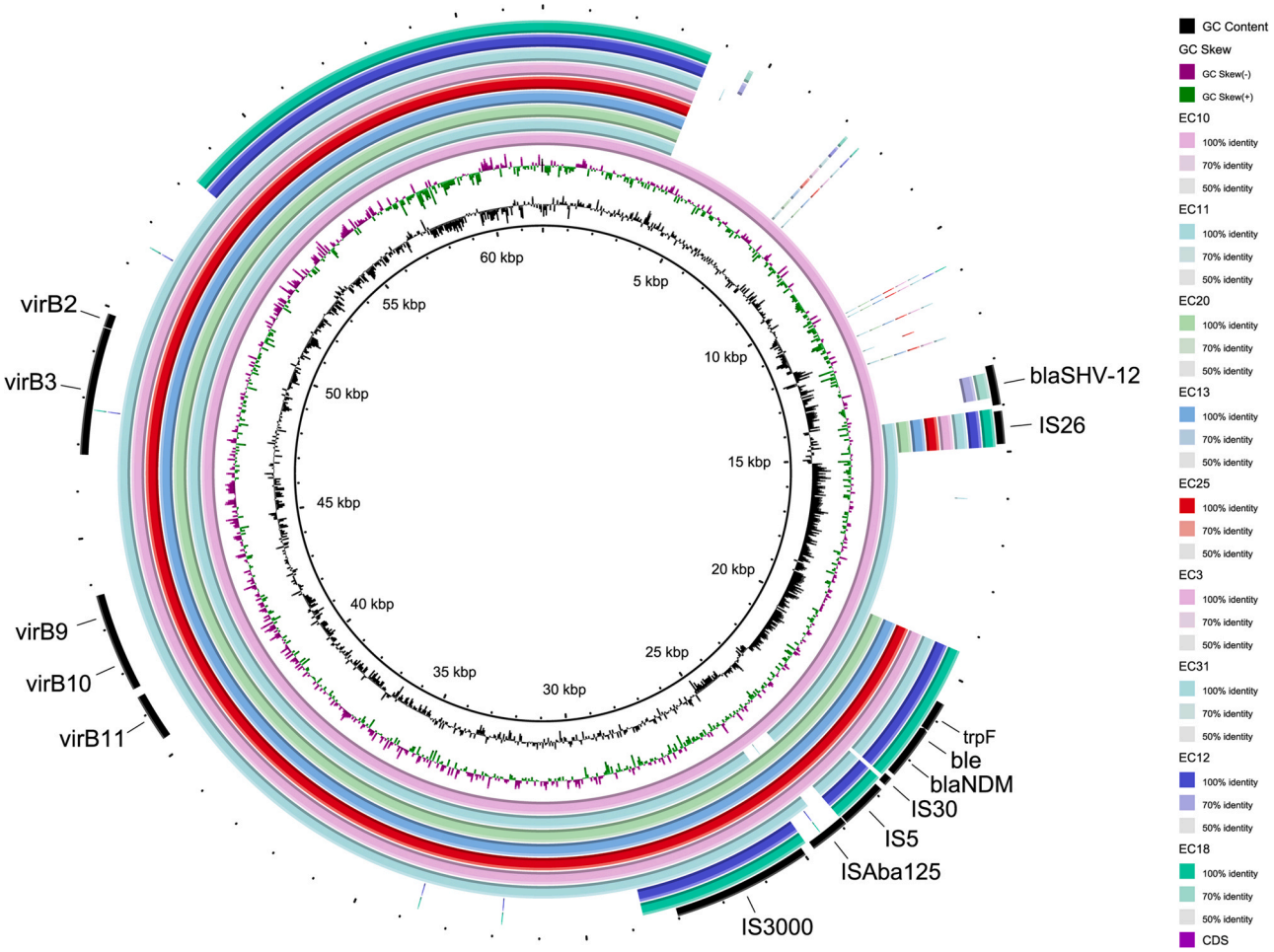

Fig. 4. Plasmid backbone comparisons of IncX3 plasmids with various sizes. Plasmid information is presented in Table 3. Antibiotic resistance genes, ISs and conjugation-associated elements are indicated in black.

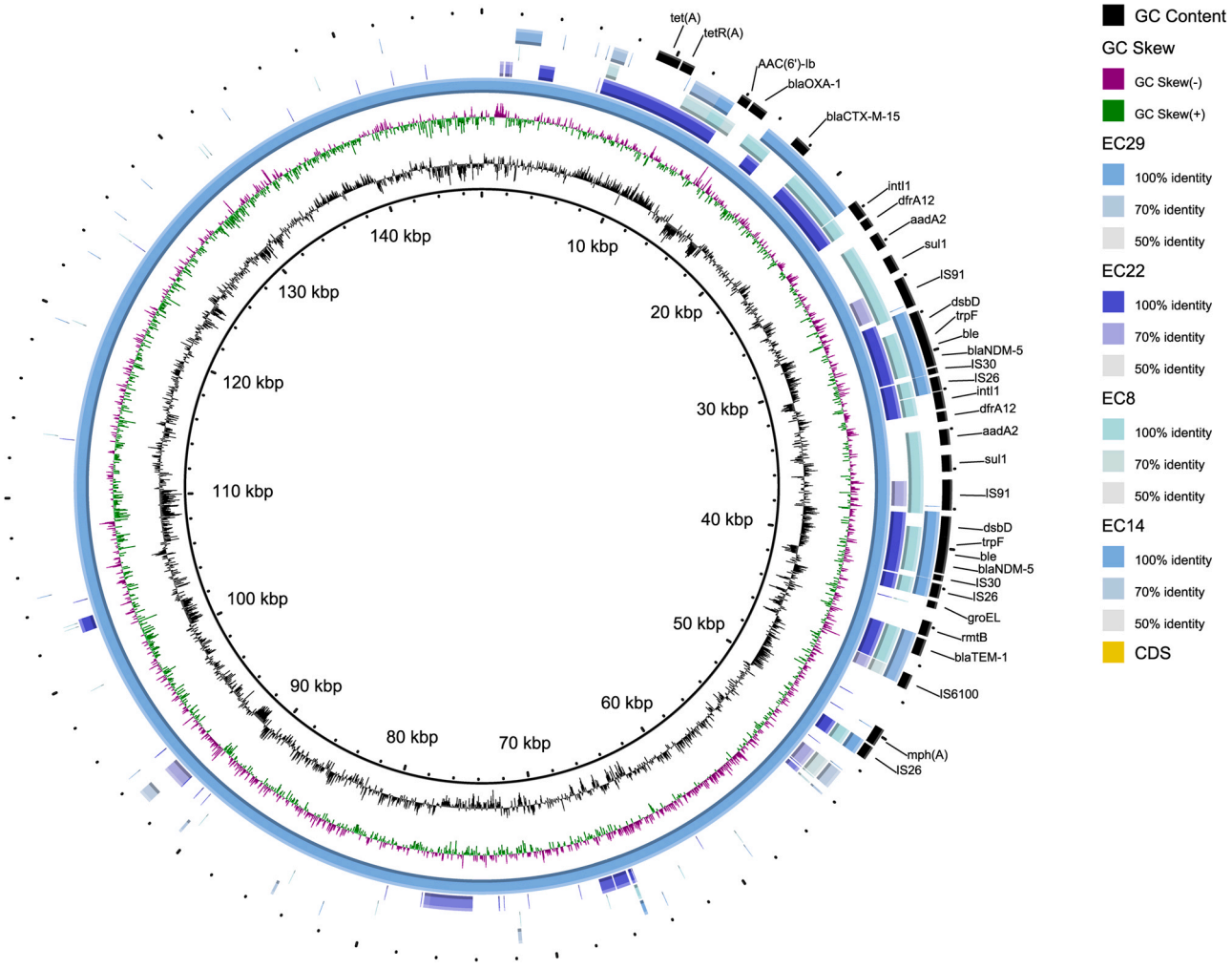

Fig. 5. Plasmid backbone comparisons of the IncA/C2, IncL/M, IncX3/IncFIB, and IncFIA plasmids.

aminoglycoside resistance genes (*aac(6′)-Ib*, *aadA2*, *rmtB*), β-lactam resistance genes ($bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, $bla_{SHV\text{-}12}$, $bla_{TEM\text{-}1B}$), sulfonamide resistance genes (*sul1*), trimethoprim resistance genes (*dfrA12*), tetracycline resistance genes (*tet(A)*) and macrolide resistance genes (*mph(A)*). Despite being broad host plasmids, IncL/M-, IncA/C2-, and IncFIA-type plasmids have larger plasmid structures, but they are less effectively conjugated.

To compare the differences in the surroundings of the bla_{NDM} gene on different plasmids, the structures of the representative plasmids of different replicon types were compared, as shown in Fig. 6. The structure surrounding the bla_{NDM} gene is relatively conserved and mainly contains the following structures: IS*3000*-IS*Abal25*-IS*5*-bla_{NDM}-ble_{MBL}-*trpF*-*dsbC*-IS*26*. However, the plasmid backbone structure is highly variable. In addition, we discovered two copies of the $bla_{NDM\text{-}5}$ gene in the pEC29-NDM-5 plasmid, with an identical structure around the gene and the complete sequence of the class 1 integron.

4. Discussion

In China, members of the family Enterobacterales are the most prevalent clinical pathogens, with *E. coli* continuously being the most common [28,29]. According to the bacterial resistance surveillance data of CHINET, the isolation rate of carbapenem-resistant *E. coli* (CREC) is increasing yearly, from 0.3 % in 2005 to 2.0 % in 2021. Most of these strains are multidrug-resistance or extensively drug resistance, and they can spread resistance genes between strains via plasmids, making infection treatment difficult and morbidity and mortality rates high; furthermore, this spread poses a serious challenge to anti-infection treatment and infection control worldwide. In this study, 795 *E. coli* strains isolated from a tertiary hospital in China over a period of two years were analysed and a total of 27 CREC strains were identified. The resistance gene screen revealed that 17 strains carried the $bla_{NDM\text{-}5}$ gene, 5 strains carried the $bla_{NDM\text{-}1}$ gene, 1 strain carried the $bla_{NDM\text{-}7}$ gene, and the remaining 4 strains carried the $bla_{KPC\text{-}2}$ gene, indicating that the production of NDM-type carbapenemases is the main mechanism of carbapenem resistance in *E. coli*, with the NDM-5 subtype being the most common.

All 23 NDM-producing *E. coli* strains were multidrug-resistant and they carried multiple antimicrobial resistance genes, the majority of which were located on plasmids; these strains also showed resistance to all antibiotics except tigecycline, colistin, and cefiderocol. According to our findings, tigecycline, colistin, and cefiderocol can be used to treat infections caused by CREC. Notably, CREC strains carrying both the plasmid-mediated carbapenem resistance gene bla_{NDM} and the colistin resistance gene *mcr-1* or the tigecycline resistance gene *tet*(X4) have been identified, so it is critical to pay close attention to clinical CREC transmission when in combination with other resistance genes [30–32].

Prolonged hospitalization, an immunocompromised status, older age along with severe underlying disease, prolonged use of broad-spectrum antibiotics, and invasive procedures such as deep venous cannulation and tracheotomy have all been identified as risk factors for CRE colonization and infection [33]. All CREC-positive patients in

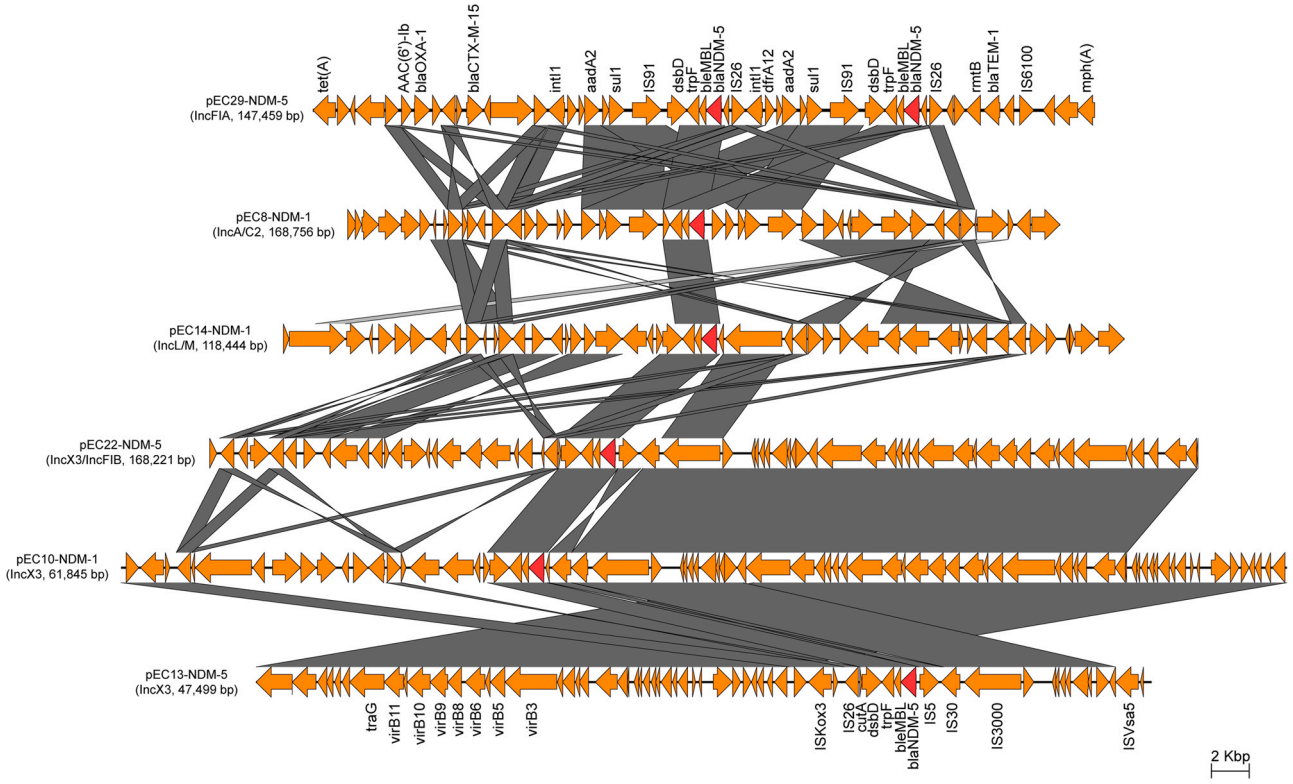

Fig. 6. Alignment of genetic surroundings of the bla_{NDM} gene on different plasmids. The CDS coding region is indicated by yellow arrows, while bla_{NDM} is indicated by red arrows.

this study were initially admitted to the hospital with a variety of underlying conditions, including intracranial injury, cerebral hemorrhage, and cerebral infarction in more than half of the patients. The vast majority of these strains (20/23, 86.96%) were isolated from urinary and respiratory specimens, which may be related to the fact that most patients with CREC infections are critically ill and frequently receive treatment measures such as catheters and mechanical ventilation. Notably, five of the 23 bla_{NDM}-positive strains in this study were isolated from individuals under the age of 35 who were admitted for intracranial trauma or injury and later acquired a carbapenem-resistant *E. coli* infection following necessary surgical treatment (e.g., traumatic brain surgery).

According to previous studies, NDM-producing *E. coli* cover a wide range of sequence types, with ST167 being found in several countries (Korea, India, South Africa, Japan, USA and Switzerland) [34,35]. A multicentre study in China found that ST167 was the most common clonal lineage of NDM-producing *E. coli*, followed by ST410 [15]. Our findings revealed that among the 23 strains of NDM-producing *E. coli*, there were nine different sequence types, with ST410 being the most abundant, followed by ST167. Our PFGE and phylogenetic analysis results indicate a hospital epidemic of a nosocomial clone of NDM-5-producing *E. coli* ST410, which warrants further investigation. Furthermore, our findings indicated that the PFGE, cgSNP, and cgMLST results were consistent, but the resolution of cgSNP and cgMLST was higher than that of PFGE.

All plasmids carrying the bla_{NDM} gene, including those containing the bla_{NDM-1}, bla_{NDM-5}, and bla_{NDM-7} genes, were successfully conjugated into *E. coli* J53. The S1-PFGE results revealed that the bla_{NDM} gene, which was carried by all 23 strains of NDM-producing *E. coli*, was located on the plasmid. Whole-genome sequencing revealed a variety of plasmid replicon types carrying the bla_{NDM} gene, including IncX3, IncFIA, IncX3/IncFIB, IncL/M, and IncA/C2; this result indicated that multiple types of plasmids can mediate horizontal transmission of the bla_{NDM} gene, with IncX3 being the most common. The fact that IncX3-type plasmids had a higher conjugation efficiency than other plasmids may be the reason why they serve as the dominant plasmid for the spread of the bla_{NDM} gene in *E. coli*. The bla_{NDM}-carrying plasmid is made up of a backbone region that encodes essential proteins for plasmid replication, transfer, and virulence and a variable region that contains antimicrobial resistance genes. Whole-genome sequencing of the bla_{NDM}-carrying plasmid revealed that the GC content of the variable region sequence was significantly higher than that of the backbone region, indicating that the region is of exogenous origin and that the variable region carrying the bla_{NDM} gene can be transferred.

Furthermore, we identified two copies of the bla_{NDM-5} gene in the pEC29-NDM-5 plasmid, with identical structures around the gene and the complete sequence of the class 1 integron. The result of the antimicrobial susceptibility testing of cefiderocol indicated that the MIC was 4 mg/L, which is higher than isolates carrying the single copy of bla_{NDM-5} gene. Simner et al. also discovered that increased copy number of bla_{NDM-5} is associated with progressive development of cefiderocol resistance in *E. coli* during therapy [36]. The emergence of multiple copies of the bla_{NDM-5} plasmid and strains may thus pose a threat to cefiderocol use and this phenomenon should be taken seriously.

5. Conclusions

Our findings revealed the main mechanism of carbapenem resistance in *E. coli* is the production of NDM-type carbapenemase, with the NDM-5 being the most prevalent. The bla_{NDM} genes are all located on conjugative plasmids ranging in size from 20 kb to 200 kb, with the IncX3-type plasmid being a key factor in the horizontal transmission of bla_{NDM}. The bla_{NDM} gene is more likely to spread horizontally between different plasmids because of the highly variable nature of the plasmid backbone structure, but the structure surrounding the bla_{NDM} gene is relatively conserved. The emergence of two copies of the bla_{NDM-5} gene on a single plasmid poses a threat to the use of the novel siderophore cephalosporin.

Funding

This work was supported by the National Natural Science Foundation of China (82172314) and the Zhejiang Provincial Medical and Health Science and Technology plan (2022KY022 and 2023KY484).

CRediT authorship contribution statement

HF designed the experiments. XJ and GH performed the experiments and were the major contributors in writing the manuscript. LLR analyzed the data. All authors read and approved the final manuscript.

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Zhejiang Provincial People's Hospital. Written informed consent from the patients was exempted by the Ethics Committee of Zhejiang Provincial People's Hospital because the present study only focused on bacteria.

Data availability

All data generated or analyzed during this study are included in this manuscript. Interested readers can contact the corresponding author for further information.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.01.004.

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