

Emergence of Tigecycline and Carbapenem-Resistant *Citrobacter freundii* Co-Carrying *tmexCD1-toprJ1*, *bla_{KPC-2}*, and *bla_{NDM-1}* from a Sepsis Patient

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Purpose: This research aims to profile ten novel strains of carbapenem-resistant *Enterobacteriaceae* (CRE) co-carrying *bla_{KPC}* and *bla_{NDM}*.

Methods: Clinical CRE strains, along with corresponding medical records, were gathered. To ascertain the susceptibility of the strains to antibiotics, antimicrobial susceptibility tests were conducted. To validate the transferability and cost of fitness of plasmids, conjugation experiments and growth curves were employed. For determining the similarity between different strains, ERIC-PCR was utilised. Meanwhile, whole genome sequencing (WGS) was performed to characterise the features of plasmids and their evolutionary characteristics.

Results: During the course of this research, ten clinical CRE strains co-carrying *bla_{KPC}* and *bla_{NDM}* were gathered. It was discovered that five out of these ten strains exhibited resistance to tigecycline. A closer examination of the mechanisms underlying tigecycline resistance revealed that *tmexCD1-toprJ1*, *bla_{KPC-2}*, and *bla_{NDM-1}* existed concurrently within a single *Citrobacter freundii* strain (CF10). This strain, with a minimum inhibitory concentration (MIC) of 32 mg/L to tigecycline, was obtained from a sepsis patient. Furthermore, an investigation of genome evolution implied that CF10 belonged to a novel ST type 696, which lacked analogous strains. Aligning plasmids exposed that similar plasmids all had less than 70% coverage when compared to pCF10-*tmexCD1*, pCF10-*KPC*, and pCF10-*NDM*. It was also found that *tmexCD1-toprJ1*, *bla_{KPC-2}*, and *bla_{NDM-1}* were transferred by *Tn5393*, *IS5*, and *Tn6296*, respectively.

Conclusion: This research presents the first report of coexistence of *tmexCD1-toprJ1*, *bla_{KPC-2}*, and *bla_{NDM-1}* in a carbapenem and tigecycline-resistant *C. freundii* strain, CF10.

Importance: Tigecycline is considered a “last resort” antibiotic for treating CRE infections. The ongoing evolution of resistance mechanisms to both carbapenem and tigecycline presents an alarming situation. Moreover, the repeated reporting of both these resistance mechanisms within a single strain poses a significant risk to public health. The research revealed that the genes *tmexCD1-toprJ1*, *bla_{KPC-2}*, and *bla_{NDM-1}*, which cause carbapenem and tigecycline-resistance in the same strain, were located on mobile elements, suggesting a potential for horizontal transmission to other Gram-negative bacteria. The emergence of such a multi-resistant strain within hospitals should raise significant concern due to the scarcity of effective antimicrobial treatments for these “superbugs”.

Keywords: *bla_{KPC}*, *bla_{NDM}*, carbapenem-resistant *Enterobacteriaceae*, tigecycline-resistance, *tmexCD1-toprJ1*

Introduction

Carbapenem-resistant *Enterobacteriaceae* (CRE) infections are highly prevalent in China, with *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, and *Citrobacter freundii* being the primary culprits.¹ The fatality rate for patients

suffering from carbapenem-resistant *K. pneumoniae* (CRKP) infections is reported to be 24.3%, and this figure rises to 40% for those patients with lower respiratory tract infections.² Strains co-carrying KPC (*Klebsiella pneumoniae* carbapenemase) and NDM (NewDelhimetallo- β -lactamase) have been reported globally in recent years, including instances in India,³ Turkey, Brazil,⁴ and China.⁵ Previous reports have described *C. freundii* strains, specifically WCHCF65 from hospital sewage and HN380 from clinical urine, co-carrying *bla*_{KPC} and *bla*_{NDM}.^{6,7} The occurrence of both *bla*_{KPC} and *bla*_{NDM} in a single strain is a matter of serious concern due to the associated higher-level carbapenem resistance and the acceleration of carbapenemase gene transmission.⁸

Tigecycline is generally regarded as the antimicrobial of “last resort” for CRE infections.⁹ Nonetheless, resistance to tigecycline has emerged since its approval, with frequent reports within *Enterobacteriaceae*.¹⁰ Previous research has indicated that chromosomal mutations and overexpression of efflux pumps were closely tied to tigecycline resistance.¹¹ In addition, a plasmid-mediated resistance-nodulation-division (RND) family multidrug efflux pump gene cluster, *tmexCD1-toprJ1*, has recently been identified and characterized.¹² Currently, *tmexCD1-toprJ1*-like-positive *K. pneumoniae* strains have predominantly been found in China and Vietnam.^{13,14} While they are commonly located in animals, food, and the environment, these strains are rarely isolated from patients.^{15,16} An increased *tmexCD1-toprJ1* presence has been noted to elevate the minimum inhibitory concentration (MIC) of *K. pneumoniae*, *E. coli*, and *Salmonella* against tetracyclines (including tigecycline and eravacycline), quinolones, cephalosporins, and aminoglycosides between four to thirty-two fold.¹²

Apart from the efflux pumps' contribution to tigecycline resistance, the proliferation of mutant *tet(A)* genes is of concern, given their potential for increasing tigecycline resistance in *K. pneumoniae* upon transmission.¹⁷ The *tet(A)* gene, associated with the major facilitator superfamily (MFS) family efflux pump, could escalate the accumulation of tigecycline, thus enhancing resistance. Notable in various sources such as food, clinical samples, environmental components and human microflora from diverse countries,^{18–20} any source could harbour the mutated *tet(A)* gene.^{21,22} Consequently, the acquisition of a *tmexCD1-toprJ1*-positive plasmid by *Enterobacteriaceae* species, particularly CRE, could generate pan-drug-resistant strains, potentially leading to untreatable infections. Hence, curbing the emergence and spread of tigecycline-resistance CRE is an epidemiological urgency.

The molecular epidemiological characteristics of ten CRE strains co-carrying *bla*_{KPC} and *bla*_{NDM} have been described herein. One strain was discovered to contain the mobile tigecycline resistance gene, *tmexCD1-toprJ1*.

Materials and Methods

Samples and Clinical Information Collection

Ongoing surveillance of CRE in China enabled the collection of clinic CRE isolate samples from the First Affiliated Hospital of Chongqing Medical University from January to December 2021. The Vitek 2 Compact (BioMérieux) was employed to confirm these isolates. The CRE collection criteria required an MIC ≥ 4 mg/L for imipenem or meropenem. PCR and Sanger sequencing confirmed all isolates co-carrying *bla*_{KPC} and *bla*_{NDM}. All PCR primers are provided in [Table S1](#). Standard and predetermined case report forms collected patient clinical information, including demographics, sample source and type, underlying diseases, clinical presentations, antimicrobial therapy, and outcomes.

Antimicrobial Susceptibility Testing (AST) and String Test

The broth microdilution method, as per Clinical and Laboratory Standards Institute (CLSI) guidelines,²³ assessed antimicrobial agent susceptibility using aztreonam, ceftazidime, imipenem, ceftriaxone, cefepime, cefoxitin, meropenem, amikacin, tigecycline, levofloxacin, and colistin. The tigecycline breakpoints with an MIC ≥ 4 mg/L for *Enterobacteriaceae* were interpreted based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (<http://www.eucast.org/Clinical-breakpoints/>). *E. coli* ATCC25922 served as a control. The string test validated the hypermucoviscous phenotype of *K. pneumoniae*.²⁴ In brief, the test is considered positive if a bacteriology inoculation loop or needle can create a viscous string longer than 5 mm by stretching bacterial colonies on an agar plate.

ERIC-PCR and Tigecycline-Resistant Mechanism

The Enterobacterial Repetitive Intergenic Consensus (ERIC) methodology serves as a molecular tool employed in epidemiological investigations and bacterial species genotyping.²⁵ The ERIC-PCR assay was conducted with the PCR primers specified in [Table S1](#), as described earlier. The unweighted pair-group method with an arithmetic mean evaluated genetic diversity and categorised isolates with $\geq 80\%$ identity into a single cluster.²⁶ PCR and Sanger sequencing detected and confirmed tigecycline-resistant genes, *ramR*, *acrR*, *rpsJ*, *oqxR*, *tet(A)*, *tmexCD1-toprJ1*, and *tet(X)*. Mutation analysis aligned sequences from each sample against references from wild-type strains, such as *E. coli* plasmid RP1 (X00006) for the *tet(A)* genes and *K. pneumoniae* MGH78578 (CP000647) for the remaining genes.

Conjugation Experiments and Transconjugant Characteristics

The plasmid-encoded *bla*_{KPC}, *bla*_{NDM}, and *tmexCD1-toprJ1* genes' transferability was ascertained via conjugation assays. In these assays, KPC-NDM-CRE (CRE co-harboring *bla*_{KPC} and *bla*_{NDM}) strains acted as donors, and the rifampin-resistant *E. coli* 600 strain was the recipient. Mueller-Hinton agar (MHA, Oxoid) plates with rifampin (200 mg/L) and either meropenem (2 mg/L) or tigecycline (2 mg/L) were used to select transconjugants. Subsequent AST and PCR amplification confirmed successful plasmid transfer. Plasmid fitness cost was gauged using a growth curve assay,²⁷ wherein bacteria were diluted overnight with 1:100 Luria-Bertani broth and cultured at 37 °C and 200 rpm. The OD600 value was tested hourly, with the process repeated thrice.

Analyzing the Prevalence of Reported Strains Co-Carrying *bla*_{KPC} and *bla*_{NDM}

The prevalence of reported strains co-carrying *bla*_{KPC} and *bla*_{NDM} was analysed by collecting relevant information from PubMed and National Center for Biotechnology Information (NCBI) databases. These included species, temporal data, geographical location, source of specimens, molecular typing, plasmid typing, and other characteristics.

Whole Genome Sequencing (WGS) and Plasmid Analysis

The CF10 strain genome was sequenced using the third-generation Nanopore platform (Oxford, UK) and the second-generation Illumina X Ten sequencing platform (Illumina, San Diego, CA). The other nine CRE strains were sequenced using the second-generation MGI2000 platform (Genomics, China). The software fastp version 0.23.0 (<https://github.com/OpenGene/fastp>) was employed for data cleaning, with reads of low sequencing quality, higher N proportions, and smaller lengths post-quality pruning removed. SPAdes v.3.5.0 (<http://cab.spbu.ru/software/spades/>) assembled the trimmed second-generation sequencing data. Prokka v.1.10 (<https://github.com/tseemann/prokka>) was utilised for gene prediction, while ResFinder v.4.1 (<http://genepi.food.dtu.dk/resfinder>) predicted antibiotic resistance genes. Virulence genes were predicted using the virulence factor database (VFDB; <http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi>). The MLST v.2.0 (<https://pubmlst.org>) and PlasmidFinder v.2.1 (<https://cge.food.dtu.dk/services/PlasmidFinder/>) were used to analyse multilocus sequence typing (MLST) and plasmid incompatibility types. ICEberg v.2.0 (<http://db-mml.sjtu.edu.cn/ICEberg/>) predicted mobile elements like insertion sequences and transposons. A complete *K. pneumoniae* K-locus reference database can be found at <https://github.com/katholt/Kaptive>. The CGView server (<http://cgview.ca/>) visualised the plasmid's circular representation,²⁸ while BLAST v.2.0 performed linear alignment and comparison of sequences, the results of which were visualised by Easyfig v.2.2.2.²⁹

Evolution Analysis

To conduct evolution analysis, a search for homologous sequences in the NT and PLSDB databases identified 20 plasmids resembling pCF10-tmexCD (with a bidirectional threshold coverage of $\geq 45\%$), 28 plasmids resembling pCF10-NDM (with a coverage of $\geq 48\%$), and 12 plasmids resembling pCF10-KPC (with a coverage of $\geq 60\%$). This was followed by the generation of phylogenetic trees of plasmids using KSNP3, which identified single nucleotide polymorphisms (SNPs) directly based on k-mer and built maximum likelihood trees without a reference sequence. Hierarchical clustering was carried out using the Cluster package from the software package R. Minimum spanning tree analysis was executed using PHYLOVIZ. The evolutionary pathway was constructed using the phylogenetic trees'

genetic distance and sequence coverage. The R ggtree package generated the phylogenetic tree, and the pathway was visualised using a scatter plot (R language).

Ethical Approval Statements

This study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Evaluation Committee and the Biomedical Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (2022-0310). Given the study's retrospective and anonymous nature, the Ethics Committee deemed written informed consent from participants unnecessary.

Results

Clinical Characteristics of KPC-NDM-CRE Strains

This study confirmed ten strains co-harboring *bla*_{KPC} and *bla*_{NDM}. These comprised three *K. pneumoniae*, five *E. coli*, one *E. cloacae*, and one *C. freundii* strains. Of these, four strains were isolated from the intensive care unit (ICU), and two out of ten patients had sepsis. Additionally, two of the three patients infected with CRKP succumbed to the infection. Table 1 presents these clinical characteristics.

Antimicrobial Susceptibility Profiles and String Test

Table S2 reveals that all strains exhibited resistance to cephalosporins, penicillin, and β -lactam compounds. However, all strains were susceptible to aztreonam/avibactam and colistin. The EL82 strain was the only one susceptible to carbapenem, while five strains resisted tigecycline. The CF10 strain demonstrated resistance to tigecycline, with a MIC value of 32 mg/L. All *K. pneumoniae* strains tested negative in the string test.

Microbiological Characteristics

Molecular typing (Figure 1a) classified *K. pneumoniae* into two MLSTs and two capsule antigen serotypes, and *E. coli* into three MLSTs and three serotypes. *K. pneumoniae* and *E. coli* commonly exhibited ST11 and ST410 types. *K. pneumoniae* commonly presented with the capsule antigen serotype KL64. *E. cloacae* and *C. freundii* were members of ST50 and ST969, respectively, with ST696 emerging as a new MLST type for *C. freundii*. ERIC-PCR analysis of genetic relationships disclosed identities ranging from 18% to 85% between the ten strains. Intriguingly, KP21 and KP36 displayed the highest similarity at 85%.

While the NDM plasmids were successfully transferred by two strains (EC39 and EC42), the KPC plasmids could not be transferred by conjugation. The EC42T-NDM transconjugant was susceptible to meropenem and imipenem, contrasting with the resistance exhibited by EC42 (Table S3). The fitness cost of acquiring the *bla*_{NDM} plasmids was also evaluated. Interestingly, no significant difference in growth rates was observed between the recipient strain *E. coli* 600 and the transconjugants bearing NDM plasmids (Figure 1c).

Identification of Antimicrobial Resistance Genes and Virulence Genes

WGS data were employed to predict the antimicrobial resistance genes and virulence genes of the ten KPC-NDM-CRE isolates, which were depicted in Figure 1a and b. Apart from the high frequency and diversity of resistance genes, the three *K. pneumoniae* and five *E. coli* strains also exhibited a substantial number of virulence genes. The distribution of resistance genes is outlined in Table S4. Notably, the *iutA* gene recorded the highest occurrence rate of virulence genes, followed by *iucABCD*, *fimA*, and *fimH*. Table S5 summarised the results of the mutation sites of the five tigecycline-resistant CRE isolates. The *tet(A)* variants with type 1 mutations (I5R, V55M, I75V, T84A, S201A, F202S, and V203F) were found in all tigecycline-resistant isolates.²¹ A93V, classified as a type 2 mutation, was detected in KP36 and EC85. Interestingly, CF10 had two copies of *tet(A)* genes, and the mutations in one of the copies included the type 1 mutations along with 20 other mutation sites. Two isolates revealed nucleotide changes in *ramR* (A19V) compared to that of the reference strain MGH78578 (CP000647). The *tet(X)* was absent in five strains, but *tmexCD1-topJ1* was detected in CF10.

Table 1 Clinical Characteristics of Patients with KPC-NDM-CRE

Variables/ Patients	KP19	KP21	KP36	EC39	EC42	EC85	EC88	EC89	EL82	CF10
Species	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. cloacae</i>	<i>C. freundii</i>
Age	34	50	89	54	53	20	57	68	69	59
Gender	Male	Female	Male	Male	Female	Male	Male	Female	Female	Male
District	Rongchang	Liangping	YuBei	Jiangbei	Beibei	Yuyang	Main city	Chengdu	Tongliang	Banan
Ward	ICU	ICU	NeuroSurgery	Surgery	Gynecology	Hematology	ICU	Gynecology	Orthopedics	ICU
Underlying conditions	N/A	Intestinal-diseases, gallbladder-stones	Hypertension	Hypertension	N/A	N/A	Hypertension, diabetes	N/A	N/A	N/A
Specimen type	Catheter blood	Bronchoscopy- lavage fluid	Phlegm	Secretions	Urine	Rectal swab	Secretions	Seroperitoneum	Drainage tube	Peritoneal lavage fluid
Infection type	Sepsis	Abdominal infection	Lung infection	Lower extremity infection	Urinary Tract Infection	Intestinal infection	Lung infection	Abdominal infection	N/A	Sepsis
Prior antibiotic usage within 30 days	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Invasive procedures	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Therapeutic antimicrobial usage	IMP, AMK, TGC, SCF	IMP, TGC, TZP	CZ	P, TZP, TGC	FOX	MEM, TGC, MXF,	AMK, TEC, SCF, IMP, LZD, VA, MEM	SCF, IMP, LEV, TGC, CAZ, MXF	FOX, SCF, IMP, AMK	IMP, TGC, MEM, CAZ/AVI, LEV, TZP
Length of stay (days)	21	24	15	16	12	22	23	23	104	18
Outcome	Recovered	Died	Died	Recovered	Recovered	Recovered	Recovered	Recovered	Discharged	Discharged

Abbreviations: ICU, intensive care unit; SCTC, Stem Cell Transplant Center; CN, Gentamicin; FOX, Cefoxitin; SCF, Cefoperazone/Sulbactam; FEP, Cefepime; TGC, Tigecycline; AMP, Benzathinepenicillin; LVX, Levofloxacin; MEM, Meropenem; CAZ, Ceftazidime; AVI, Avibatan; CZ, ceftizoxime; IMP, Imipenem; AMK, amikacin; TZP, piperacillin/tazobactam; P, Penicillin; VA, Vancomycin; TEC, Teicoplanin; MXF, Moxifloxacin; N/A, No relevant information.

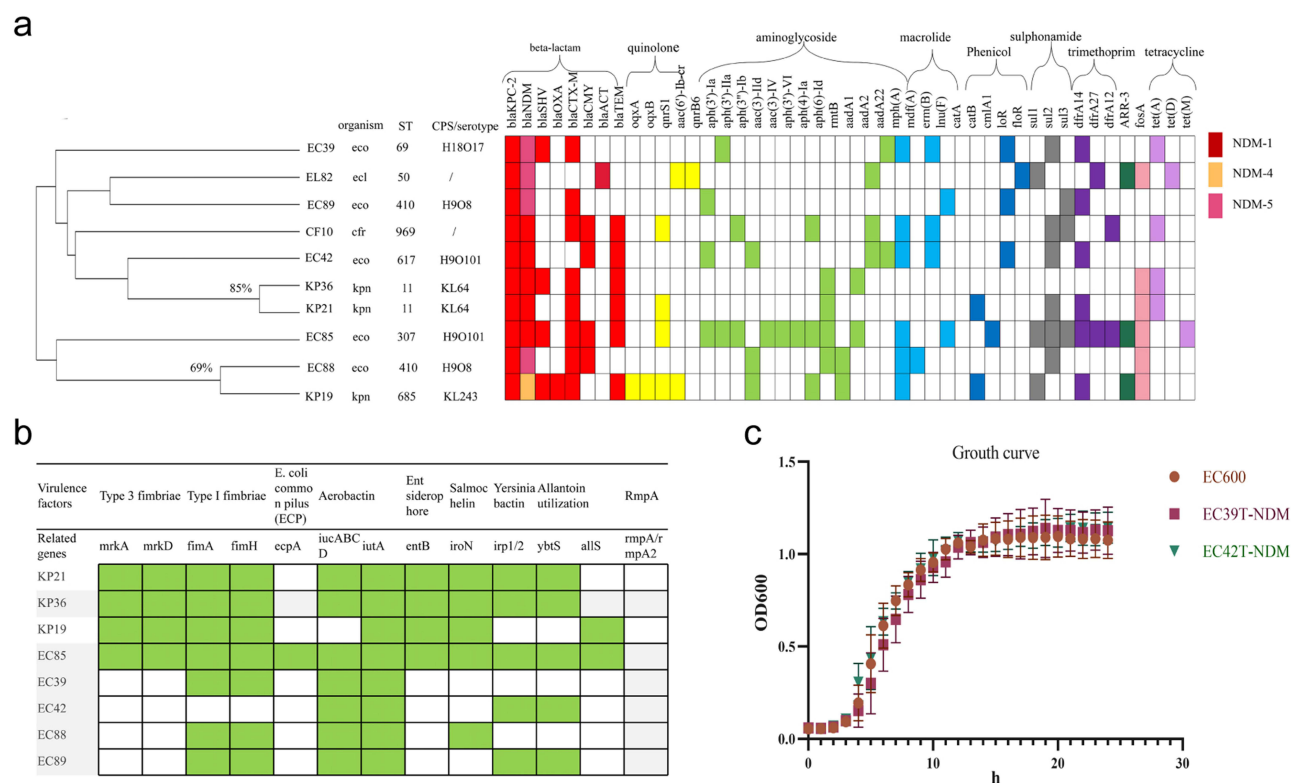


Figure 1 Microbiological characteristics of KPC-NDM-CRE. (a) On the left are the results of the ERIC-PCR clustering analysis, with numbers representing the similarity. On the right is the distribution map of antibiotics resistance genes, with coloured squares indicating positive genes. The three NDM genotypes are denoted by three colours. (b) Distribution map of the virulence genes. (c) Growth curves of transconjugants EC39T-NDM and EC42T-NDM, and the recipient strain EC600.

Reported Prevalence of Co-Carrying *bla*_{KPC} and *bla*_{NDM} Isolates

A total of 51 strains co-harboring *bla*_{KPC} and *bla*_{NDM} were collected (Table S6) from Pubmed and NCBI. Among these, ten strains of ST15 CRKP have been prevalently found in Turkey,³⁰ and three strains of ST11 CRKP were transmitted in Oman³¹ as clones. *K. pneumoniae* accounted for the highest proportion at 45% among the 51 strains, followed by *E. cloacae* at 11.8%. KPC-NDM-CRE have been reported globally, with China having the highest number of strains at 47% (Figure 2). The primary source of these bacteria was blood, potentially responsible for 16% of deaths, with most fatalities having been infected with *K. pneumoniae*. The most prevailing types of *K. pneumoniae* strains were ST11 and ST15. The strain IR98, discovered in India in 2010, carried both *bla*_{KPC} and *bla*_{NDM}, and was also tigecycline-resistant.³ Additionally, 25.5% of strains were non-susceptible to tigecycline, and potentially more as 21.5% of the strains did not have a tigecycline susceptibility result. Notably, KPC-NDM-CRE strains have been identified in hospital sewage, river sediment, vegetables, and retail food.³² Further details are provided in Table S6.

Genetic Characteristics of CF10

WGS data revealed that the CF10 strain had a chromosome of 5,010,143 bp and eight plasmids. The pCF10-tmexCD1 plasmid had a length of 212,154 bp, with a total of 186 open reading frames (ORFs) and a GC content ratio of 51.54% (Table S7). It belonged to the IncHIA+IncHIB group as it contained a replication gene *rephIA* and an additional *rephIB* gene (Figure 3a). BLASTn search analysis indicated that the pCF10-tmexCD1 plasmid had a 99.95% identity and 57% query coverage (the ratio of the query sequence length that aligned with the database sequence) with the following plasmids: pMH13-051M_1 (AP018572.2), pEC-13-33-NDM-1 (MZ836798.1), p7_SCLZS62 (CP082175.1), and pCHS4.3-1 (OL964513.1). The pCF10-tmexCD1 plasmid, interestingly, comprised a 52-kb multidrug-resistant region (MDR) flanked by multiple transposase genes, with *IS26* being the most common (Figure 4a). These resistance genes in MDR included the *tmexCD1-torJ1*, *bla*_{TEM-1B}, *aph(6')-Id*, *aph(3')-Ib*, *aadA2*, *mph(A)*, *msr(E)*, *qnrS1*, *sul2*, *sul1*, *tet(A)1*, *tet(A)2*, and *dfrA12* gene clusters.

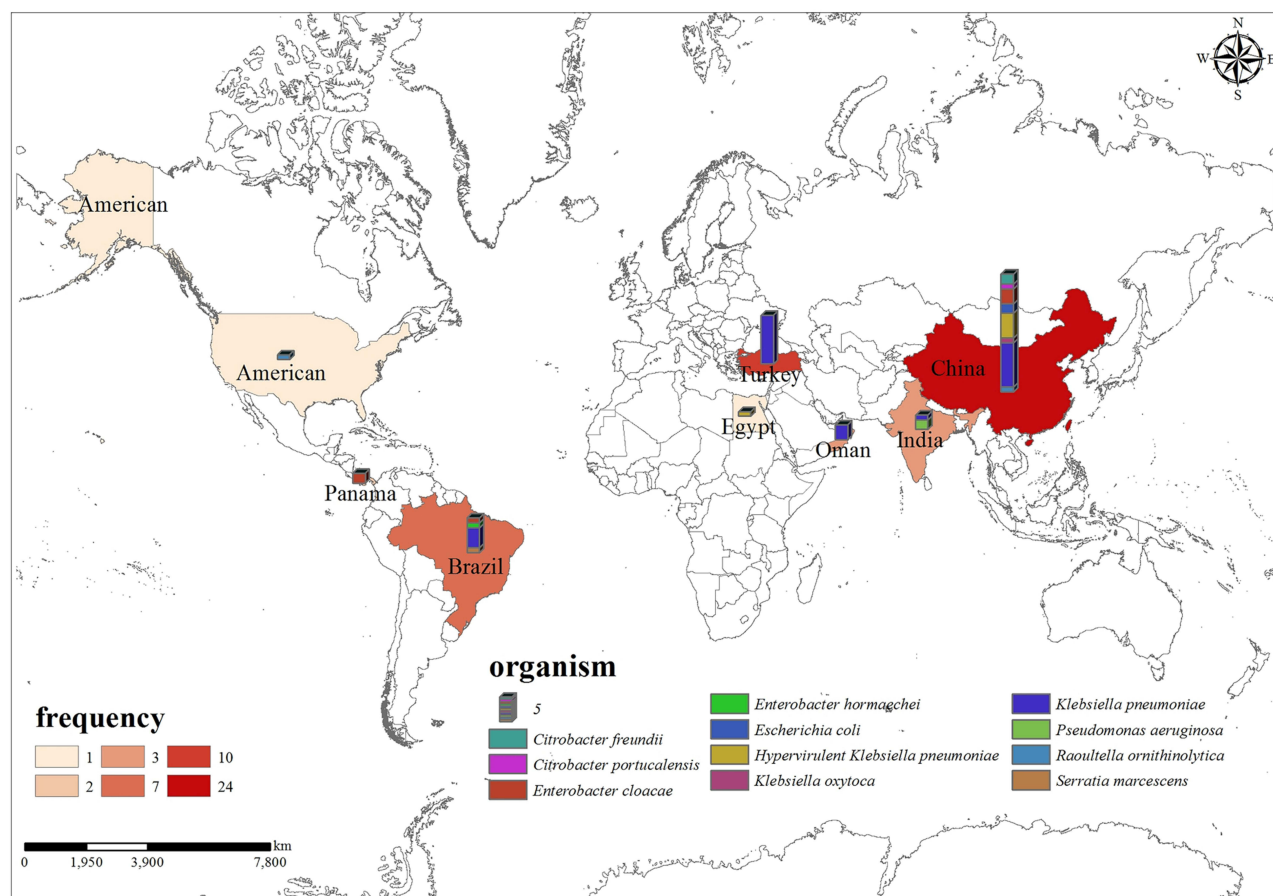


Figure 2 Prevalence and distribution of co-carrying *bla*_{KPC} and *bla*_{NDM} isolates. Different colours are used to represent different organisms and frequencies.

Furthermore, a 32-kb sequence containing the *tmexCD1-toprJ1* gene bore a strong resemblance to pHNAH8I-1 (MK347425.1) from *K. pneumoniae*, found in a chicken farm in Anhui province in 2017, with 99.96% identity and 67% query coverage. Comparative analysis of the genetic context (Figure 4b) revealed a common genetic structure, *Tn5393-int1-like-int2-like-hp1-hp2-tnfxB1-tmexCD1-toprJ1*, which was most likely derived from *Tn5393*. In addition to *Tn5393*, *IS26* also participated in the transfer of *tmexCD1-toprJ1*, *qnrS*, and *tet(A)* genes, which were located upstream of *tet(A)* and downstream of *qnrS*. In contrast to the *strA-strB* of *Tn5393-3'* retained in pHNAH8I-1, which formed the *tnpR-strA-strB* structure, a *Tn6361* remnant was found downstream of *tnpR* in pCF10-*tmexCD1*, forming the *tnpR-qnrS1-IS26* structure. The $\Delta Tn6361$ was likely acquired in pCF10-*tmexCD1* through massive recombination events occurring between the two copies of *tnpR*.

The pCF10-NDM was a 138,196-bp plasmid with an IncFII(Yp)-type replicon that contained 155 predicted ORFs and a GC content ratio of 51.49% (Table S7). Additionally, a whole plasmid BLASTn search found pCF10-NDM to have 99.96% identity and 47% query coverages with p205880-NDM in *K. pneumoniae* (MH909345.1), pKOX_NDM1 in *K. michiganensis* (JQ314407.1), and pNDM1_015096 in *K. pneumoniae* (CP043589.1). The aligned region between the pCF10-NDM and references contained both conjugation-related regions and *bla*_{NDM}-bearing MDR (Figure 3b). Almost identical to pNDM-SCNJ07 (MK933278.1) from an *Enterobacter hormagchei* isolated in Chengdu, the genetic context of *bla*_{NDM-1} between them had a 99.95% identity (Figure 4c). In both plasmids, the transposition units had been organised in an *IS5-ISEhe3-ISEhe-ISC21-groEL-groES-cutA-dsbC-trpF-ble_{MBL}-bla_{NDM-1}-ISKpn26-ISKpn26* structure. More often than not, the *bla*_{NDM-1} gene was surrounded by downstream genes (*ble_{MBL}-trpF-dsbC-cutA1-groL*) flanked by *IS5*, which implied *IS5*'s involvement in the mobilisation of the *bla*_{NDM-1} gene.

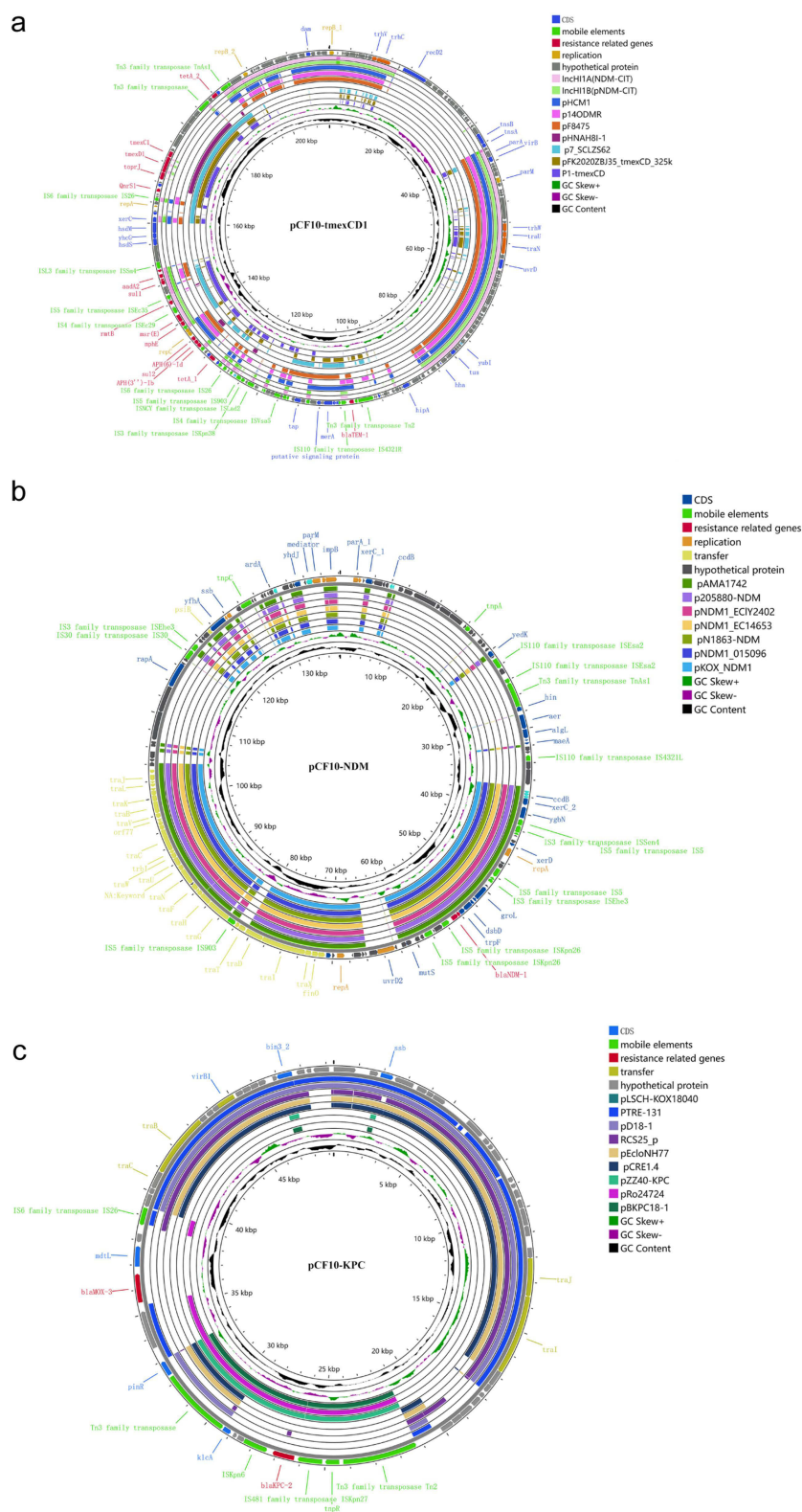


Figure 3 Comparison analysis of pCF10-tmexCD1(a), pCF10-NDM(b), and pCF10-KPC(c) plasmids with their similar plasmids in the circle map. Open reading frames (ORFs) are indicated by arrows in the outside circle. Red arrows indicate the presence of resistance genes, green arrows represent mobile elements, brown arrows depict replication genes, and grey arrows signify hypothetical proteins.

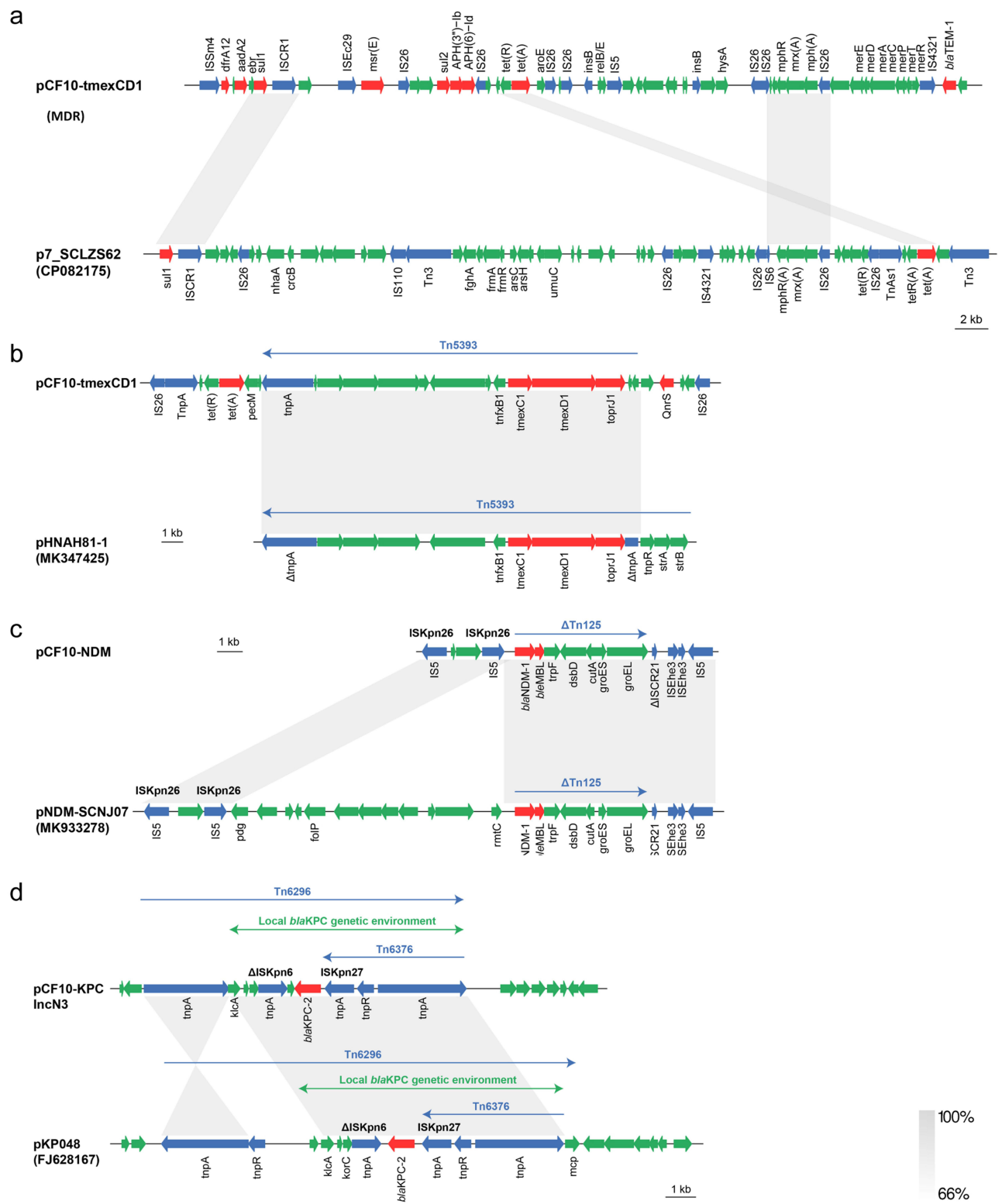


Figure 4 Genetic contexts of MDR in pCF10-tmexCD1(a), *tmexCD1-topJ1*(b), *bla_{NDM-1}*(c), and *bla_{KPC-2}* (d) genes compared with other similar sequences. Red arrows indicate the presence of resistance genes, green arrows represent mobile elements, and blue arrows signify CDS genes. The depth of the grey area represents the percentage of similarity between two sequences.

The 49-kb pCF10-KPC was identified as an IncN3-type plasmid, comprising 85 predicted ORFs and having a GC content ratio of 51.52% (Table S7). Two resistance genes (*bla*_{KPC-2} and *bla*_{MOX-3}) and five mobile elements were present in the pCF10-KPC plasmid (Figure 3c). The backbone of pCF10-KPC, when searched through BLASTn, showed 99.9% identity and 64% query coverage with pZZ40-KPC (MN891679.1), pBKPC18-1 (CP022275.1), pCRE1.4 (CP034398.1), and pD18-1 (CP022277.1). The sequences aligned between these plasmids lacked any multi-resistant regions. A high degree of homology was found in the genetic context of *bla*_{KPC-2} to the plasmid pKP048 (FJ628167) from *K. pneumoniae* (Figure 4d). This plasmid was a 12-kb fragment bordered by *Tn3* transposase with a genetic structure *tnpA-tnpR-ISKpn27-bla*_{KPC-2}-*ISKpn6-klcA-tnpA*. This unit was recognised as a transposon from the *Tn6296* group,³³ a transposon unit of the *Tn21* subfamily of the *Tn3* family, which has been largely viewed as a significant vector for *bla*_{KPC-2}, first found in plasmid pKP048.³⁴

Evolutionary Pathway of CF10

In the plasmids phylogenetic tree analysis, no similar plasmids were identified with a bidirectional coverage of $\geq 90\%$. Bidirectional coverage thresholds were subsequently adjusted to 45%, 48%, and 60% for tmexCD1, NDM, and KPC plasmids, respectively (Figure 5a–c). The MLST minimum spanning tree also demonstrated CF10's emergence as a new ST type 696, a derivative of ST95 (Figure 5d). A comparison with the WGS of CF10 from the GenBank database revealed no similar genome sequences with a query coverage $\geq 90\%$. The phylogenetic tree indicated that CF10 occupied a distinct branch (Figure 6a). Additionally, GCF_905330765_2 was identified as the genome sequence most similar to

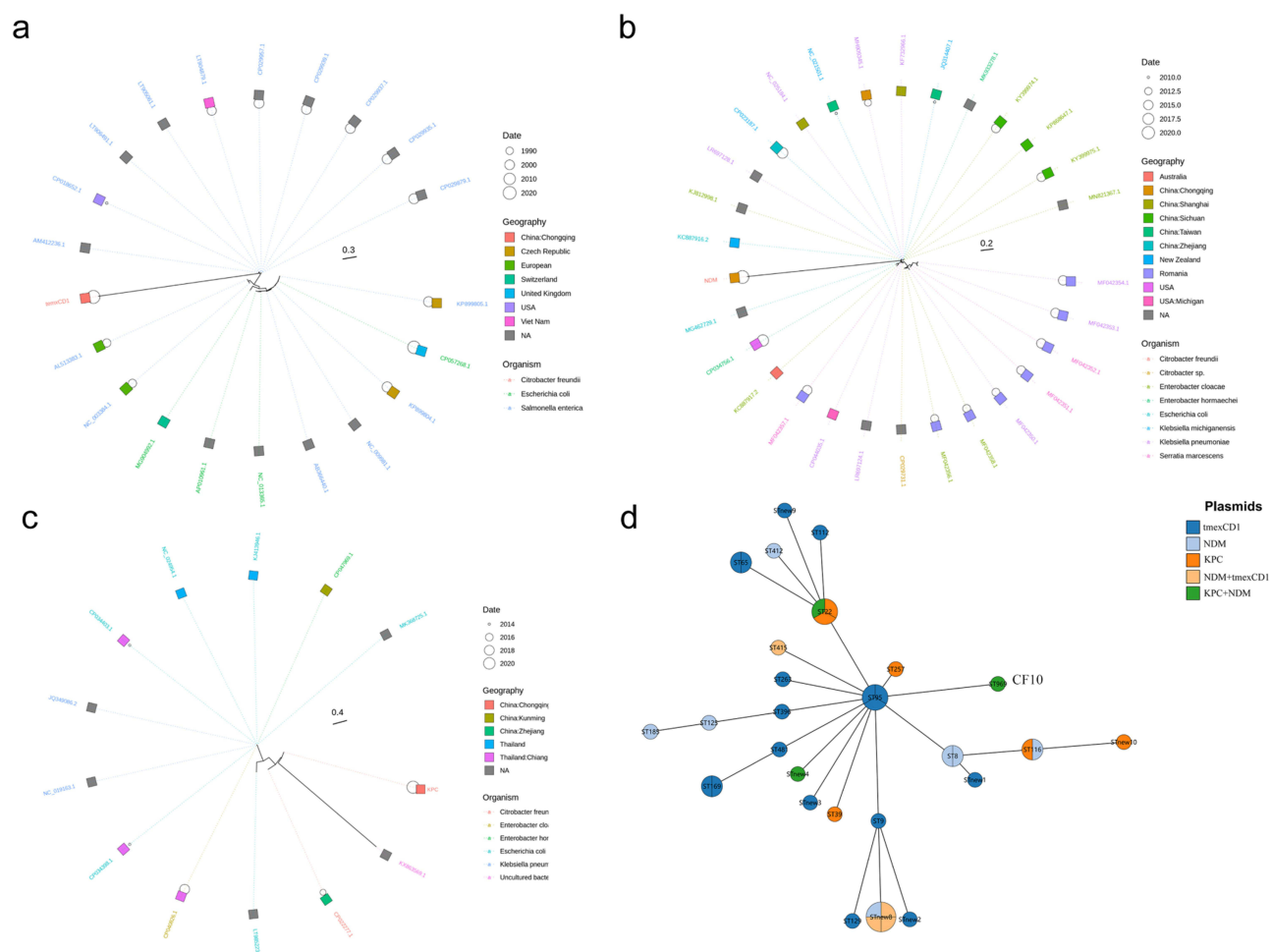


Figure 5 Phylogenetic tree plasmids and MLST minimum spanning tree. (a–c) Comparison of evolutionary characteristics of pCF10-tmexCD1, pCF10-KPC, and pCF10-NDM plasmids. The plasmid label is coloured according to the organism, a filled bar indicates geographical location, and the size of the circle represents collection date. (d) Minimum spanning tree analysis based on MLST of similar strains. The different coloured grids represent the types of resistance genes.

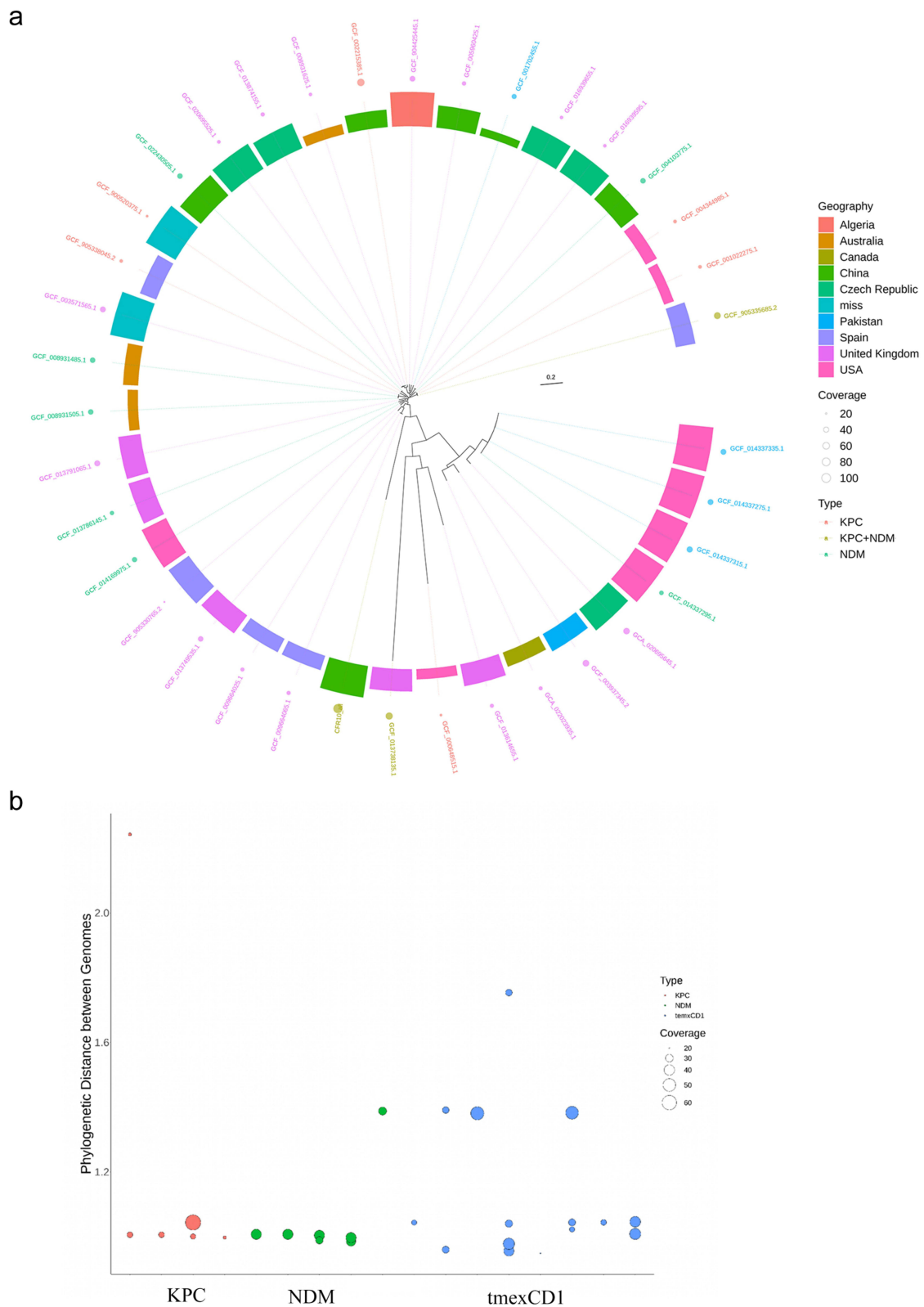


Figure 6 Evolutionary pathway of CF10. (a) Phylogenetic tree for CF10 genome with annotations: The height of the bar graph indicates the collection date, the colour of the bar graph suggests the geographical location, and the size of the circles represents the coverage of the genome bearing *bla*_{KPC-2} or *bla*_{NDM-1} genes, and the colour of the circles and the outermost circle labels symbolize the resistance genes types. (b) Scatter plot of the phylogenetic distance of genomes (y axis) versus similarity of plasmids (point size), grouped by KPC, NDM, tmexCD1 plasmids.

CF10 in the phylogenetic tree, with a difference of 104,098 SNPs between them. The phylogenetic distance evolutionary pathway showed that the *tmexCD1*, *NDM*, and *KPC* plasmids were closely associated with the CF10 genome (Figure 6b).

Discussion

The proliferation of CRE infections in hospitals has curtailed treatment alternatives, and the emergence of carbapenem resistance has posed significant clinical challenges.⁹ With tigecycline dubbed the “last resort” of defence against CRE, the combined resistance of tigecycline and carbapenem in CRE has emerged as a pressing global health issue.³⁵ In this study, the molecular epidemiological characteristics of ten CRE co-carrying *bla*_{KPC} and *bla*_{NDM} were examined. Notably, resistance to tigecycline was found in five of the ten KPC-NAM-CRE. Critically, the coexistence of *bla*_{KPC-2}, *bla*_{NDM-1}, and *tmexCD1-toprJ1* genes in a *C. freundii* strain CF10 was reported for the first time. To date, such a presence has been reported solely in *K. pneumoniae* in China. Our findings undeniably raise an epidemiological alarm, offering deeper insights into the mechanisms of tigecycline resistance and underscoring the urgent need for enhanced infection control within hospital settings.

Based on patients data, the two individuals infected with tigecycline-resistant ST11-KL64 CRKP succumbed while in the hospital, illustrating the heightened mortality rate associated with CRKP, exacerbated by tigecycline resistance. Long-standing selective pressure from tigecycline has resulted in a build-up of gene mutations and increased efflux pump gene expression, leading to decreased tigecycline susceptibility.³⁶ Previously, mutations in the *tet(A)* genes were identified as the chief contributors to tigecycline resistance among CRKP isolates.² In our investigation, all tigecycline-resistant CRE presented with type 1 mutations in the *tet(A)* variants. It has been noted that ST11-KL64 CRKP is gradually overtaking ST11-KL47 as the dominant hypervirulent CRKP clone in China, leading to heightened patient mortality.³⁷ The enhanced toxicity and tigecycline resistance of these ST11-KL64 strains amplify the death risk. The emergence and rising prevalence of ST11-K64 CRKP strains underscore the urgency for immediate containment strategies.

In our research, the genes *tmexCD1-toprJ1*, *bla*_{KPC-2}, and *bla*_{NDM-1} were identified within the singular CF10 strain but were found on separate plasmids: pCF10-*tmexCD1*, pCF10-*NDM*, and pCF10-*KPC*. It is plausible that such plasmids have become tailored to *C. freundii* over an extended time. Conjugation tests only successfully introduced the *bla*_{NDM}-bearing plasmids from EC39 and EC42. The MIC values for EC39T-*NDM* towards imipenem and meropenem decreased substantially, yet EC39T-*NDM* remained resistant. This suggests other potential resistance mechanisms, like efflux pumps and porin loss,¹¹ might be at play in EC39. In contrast, EC40T-*NDM* displayed susceptibility to both antibiotics after a 16-fold decrease in MIC values, possibly because of a truncated *bla*_{NDM} gene in EC40T-*NDM*. Moreover, the high transferability combined with the low fitness cost of *bla*_{NDM}-bearing plasmids emphasises the need to halt the proliferation of CRE strains containing both *bla*_{KPC} and *bla*_{NDM}.

Interestingly, a BLASTn analysis using the megablast function revealed that no plasmids demonstrated more than 90% identity and over 70% coverage with pCF10-*tmexCD1*, pCF10-*NDM*, and pCF10-*KPC* in the NCBI's non-redundant Nucleotide collection (nr/nt) database. Both the MLST's minimum spanning tree and the phylogenetic tree indicated that CF10 represented a novel branch. WGS data suggested that the CF10 strain, including its plasmids, did not share evolutionary origins with known strains and plasmids due to limited query coverages ($\leq 70\%$). *IS26* was found surrounding various resistance genes such as *sul2*, *qnrS*, and *tet(A)*, highlighting its role in the lateral transfer of these genes, as observed in Gram-negative bacteria.³⁸ Furthermore, it was reported that the plasmid-harboured *tmexCD1-toprJ1* gene was present in 2.5% and 52.4% tigecycline-nonsusceptible *K. pneumoniae* strains isolated from humans and animals, respectively.¹³ The current prevalence of *tmexCD-toprJ* across various clinical pathogens necessitates ongoing monitoring.³⁹ The rise of *tmexCD1-toprJ1* within CRE strains has hastened the dissemination of tigecycline resistance, underscoring the need for worldwide oversight to thwart multi-resistance transmission. Crucially, discovering strains with coexistent *bla*_{KPC} and *bla*_{NDM} in both river sediment and food products highlights a transition from the natural environment to clinical strains, expediting the lateral transfer of resistance genes and amplifying public health safety concerns.⁴⁰

This research has its limitations. The genomic and evolutionary traits of the nine sequenced CRE strains from the second generation were not studied. A more comprehensive WGS data deserves further analysis of the evolution characteristics. The impacts of tigecycline resistance gene mutations on susceptibility were not experimentally verified.

Conclusion

To conclude, this study has unveiled ten clinical CRE strains co-carrying *bla*_{KPC} and *bla*_{NDM}. We have presented the first instance of a tigecycline-resistant *C. freundii* strain, CF10, containing *tmexCD1-toprJ1*, *bla*_{KPC-2}, and *bla*_{NDM-1} plasmids. This represents a formidable “multidrug-resistant superbug” responsible for sepsis within a hospital setting.

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Disclosure

The authors report no conflicts of interest in this work.

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