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ORIGINAL RESEARCH

Molecular Characteristics and Antimicrobial Susceptibility Profiles of *bla*$_{KPC}$-Producing *Escherichia Coli* Isolated from a Teaching Hospital in Shanghai, China

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Introduction: Carbapenem-Resistant Enterobacteriaceae (CRE) has posed a significant threat to humans.The aim of this study was to investigate the molecular characteristics of *bla*$_{KPC}$-producing *Escherichia coli* in a university-affiliated tertiary hospital.

Methods: Polymerase chain reaction (PCR) and BLAST+ software were used to detect the prevalence of *bla*$_{KPC}$ in *E. coli* and *Klebsiella pneumoniae*. Whole-genome sequencing was performed for the *bla*$_{KPC}$-harboring clinical *E. coli* isolates. Antimicrobial resistance genes, MLSTs, KPC-carrying plasmid typing and genetic environment of *bla*$_{KPC}$ were analyzed. A maximum likelihood core single nucleotide polymorphism (SNP)-based phylogeny tree was constructed to determine the evolutionary relationships within this ST131 collection. Conjugation experiments were performed to determine the mobilization of *bla*$_{KPC}$. The minimal inhibitory concentrations of the common antimicrobial agents were determined using the broth microdilution method.

Results: The prevalence of *bla*$_{KPC}$ in 424 clinical *E. coli* isolates and 1636 *E. coli* strains from GenBank database were 2.2% (45/2060) whereas the detection rate of *bla*$_{KPC}$ in *K. pneumoniae* from the GenBank database was 29.8% (415/1394). The *bla*$_{KPC}$-harboring conjugants exhibited resistance to multiple β-lactams, except for cefepime-zidebactam and ceftazidime-avibactam. All *bla*$_{KPC}$-carring *E. coli* isolates were susceptible to tigecycline and polymyxin B. ST131 was the dominant sequence type of *bla*$_{KPC}$-carring *E. coli*, accounting for 40.0% (18/45). Most of the *bla*$_{KPC}$-producing ST131 *E. coli* (89.5%,17/19) belonged to clade C ST131 lineage. Genetic environment analysis revealed that 57.8% (26/45) of *bla*$_{KPC}$ gene was linked to Tn*4401*-associated structure IS*Kpn6*-*bla*$_{KPC}$-IS*Kpn7*. IncN was the most common plasmid type in KPC-producing *E. coli* whereas IncFII was the dominant plasmid type in KPC-producing *K. pneumoniae.*

Conclusion: The detection rate of *bla*$_{KPC}$ was lower in *E. coli* compared with *K. pneumonia*e. The dominant sequence and plasmid types of *bla*$_{KPC}$-harboring isolates differed between *E. coli* and *K. pneumoniae*. Further studies about the role of the defense system in acquisition of KPC-plasmids in *E. coli* will be performed to provide new insights into the low prevalence of *bla*$_{KPC}$.

Keywords: *Escherichia coli*, *bla*$_{KPC}$, carbapenemases, plasmids typing

Introduction

Escherichia coli, a member of Enterobacteriaceae family, is a prominent cause of many common bacterial infections, including urinary tract infections, bloodstream infections, diarrheal illnesses, and central nervous system infections such as neonatal meningitis.[1] The prevalence of multidrug-resistant *E. coli* poses a significant public health threat. Horizontal gene transfer (HGT), including conjugation by plasmids, transduction by bacteriophages, and natural transformation by extracellular DNA, plays a crucial role in the emergence of antimicrobial resistance.[2]

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Multiple mechanisms including the production of carbapenemases or alterations in outer membrane permeability or upregulation of efflux systems along with hyperproduction of other β-lactamases such as bla_{AmpC} result in carbapenem resistance in *E. coli*.[3] The production of carbapenemases is a major contributor to carbapenem resistance in *E. coli*. According to Ambler Classification, carbapenemases were classified into 3 groups based on their active sites: Class A (mostly KPC enzymes), Class B (metallo-β-lactamases, MBL such as VIM, NDM and IMP), and Class D (mostly OXA such as OXA-48-like and OXA-23). Class A and D enzymes have a serine-based hydrolytic mechanism, whereas class B metallo-beta-lactamases contain zinc in the active site.[4,5]

KPC emerged in the late 1990s, was first identified in 1996 in the USA.[6] KPC carbapenemases exhibit activity against a wide spectrum of β-lactams, including cephalosporins, cephamycins, aztreonam, carbapenems, and β-lactamase inhibitors.[6,7] Since their first description, KPC enzymes have spread internationally. The dissemination of KPC-producing *K. pneumoniae* is primarily associated with a single multilocus sequence type (ST), ST258, and its related variants. KPCs have been found in many gram-negative species, including Enterobacteriaceae such as *E. coli* and non-fermenters, such as *Pseudomonas aeruginosa*.[8] A nationwide survey of Carbapenem-Resistant Enterobacteriaceae(CRE) revealed that bla_{NDM} was the dominant carbapenemase genes in *E. coli* followed by bla_{KPC-2} carbapenemase.[9] It is noteworthy that research in Singapore showed that bla_{KPC} was the predominant carbapenemase type in *E. coli*, demonstrating greater dissemination potential.[10]

Given the limited epidemiological data on bla_{KPC}-producing *E. coli* in China, we investigated the molecular characteristics and antimicrobial susceptibility profiles of bla_{KPC}-harboring *E. coli* in a university-affiliated tertiary hospital in Shanghai, China.

Materials and Methods

Bacterial Strains

In this study, 424 non-duplicate *E. coli* isolates were collected from patients at a teaching hospital in Shanghai, China, for the period January to December 2017. The clinical *E. coli* isolates were identified using the Vitek2 system.

E. coli J53, an azide-resistant laboratory strain, was used as the recipient strain for the conjugation experiments. The complete whole-genome sequences of *E. coli* (1636 in total, Table S1) and *K. pneumoniae* (1394 in total, Table S2) were downloaded from the NCBI database.

Screening of bla_{KPC} Gene

Polymerase chain reaction (PCR) was used to detect bla_{KPC} in clinical isolates using the primers KPC-F(5'-TCACTGTATCGCCGTCTA-3') and KPC-R(5'-CCAACTCCTTCAGCAACA-3'). Amplification was carried out as follows: initial denaturation at 94 °C for 3 min; 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 50s; and a final elongation step at 72 °C for 5 min. BLAST + software was used to detect bla_{KPC} in the publicly available whole-genome sequences of *E. coli* and *K. pneumoniae* (Table S1 and S2).

Extraction of Genomic DNA and Whole-Genome Sequence

Genomic DNA of the clinically isolated bla_{KPC}-producing *E. coli* was extracted using a bacterial DNA kit (TIANGEN, Beijing, China). Short- and long-read whole-genome sequencing was performed by Shanghai Yuanxu Biotechnology using BGI Genomics (HiSeq X; Illumina, San Diego, CA, USA) and MinION Sequencer (Nanopore, Oxford, UK) respectively. Antimicrobial resistance genes were analyzed at the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/ResFinder/). The information on the completely sequenced bla_{KPC}-producing *E. coli* is presented in Table S3. The sequences of the 5 bla_{KPC}-positive clinical *E. coli* were listed in Supplementary data 4–8.

Multilocus Sequence Typing and bla_{KPC}-Carrying Plasmids Typing

The MLSTs of the whole genomes of *E. coli* and *K. pneumoniae* were analyzed using the MLST software (https://github.com/tseemann/mlst). Plasmid type bla_{KPC}-harboring isolates were identified using PlasmidFinder (https://cge.food.dtu.dk/services/PlasmidFinder/).

Genetic Environment of *Bla*$_{KPC}$-Positive *E. Coli*

The annotation of the 45 *bla*$_{KPC}$-postive *E. coli* was performed using Prokka software (https://github.com/tseemann/prokka). Then gggenes (https://github.com/wilkox/gggenes) was used for analyzing the flanking structure of *bla*$_{KPC}$ gene.

Phylogenomic Analysis

To determine the evolutionary relationships within this ST131 collection, a maximum likelihood core single nucleotide polymorphism (SNP)-based phylogeny tree of 114 *E. coli* isolates with ST131 and ST131* (*adk* gene 112 had 99.8% identity, ST may indicate nearest ST) was inferred with RAxML v8.2.12 (PubMed Unique Identifier 24,451,623) after the removal of recombination regions using Gubbins v2.4.1 (PMID 25414349). The resulting tree was rooted at the midpoint and visualized using iTOLs (https://itol.embl.de/).

Conjugation Assay

To determine the mobilization *bla*$_{KPC}$, conjugation experiments were performed using clinically isolated *bla*$_{KPC}$-producing *E. coli* as donor strains and *E. coli* J53 as the recipient strain. Transconjugants were selected on LB agar plates containing 50 mg/L ampicillin and 150 mg/L sodium azide. PCR amplification and sequencing of *bla*$_{KPC}$ were performed to ensure successful transfer of *bla*$_{KPC}$-bearing plasmids.

Antibiotic Susceptibility Testing

Minimal inhibitory concentrations (MICs) of common antimicrobial agents were determined using the broth microdilution method, and interpretation was recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines for 424 clinically isolated *E. coli*, KPC-positive *E. coli* and *bla*$_{KPC}$-harboring transformants. *E. coli* ATCC 25922 was used as a quality control strain for antimicrobial susceptibility testing. The interpretation was based on CLSI breakpoints for all antimicrobial agents except tigecycline and polymyxin B.[11] Tigecycline and polymyxin B MICs were interpreted using the European Committee for Antimicrobial Susceptibility Testing (EUCAST) criteria.[12]

Results

Antimicrobial Susceptibility Testing for Common Antibiotics in Clinical Isolated *E. Coli*

The susceptibility rates of 424 *E. coli* isolates to quinolone and most β-lactams were less than 40%, including ciprofloxacin, aztreonam, cefotaxime, ceftazidime, cefepime, and piperacillin, while those to imipenem and meropenem were 90.1% and 88.2%, respectively (Table 1). Low susceptibility rates were observed for piperacillin (11.3%), in contrast to the increased susceptibility observed when piperacillin was in combination with β-lactamase inhibitors, namely piperacillin-tazobactam (71.0%). Low resistance rates for amikacin, polymyxin B, and tigecycline were observed (12.0%, 0%, and 5.0%, respectively).

Prevalence of *bla*$_{KPC}$ in *E. Coli* and Genetic Environment of *Bla*$_{KPC}$-Positive *E. Coli*

The prevalence of *bla*$_{KPC}$ was 1.2% (5/424) in *E. coli* clinical isolates and 2.4% (40/1636) in *E. coli* strains in the GenBank database, whereas the detection rate of *bla*$_{KPC}$ in *K. pneumoniae* in the GenBank database was 29.8% (415/1394).

For the 45 KPC-producing *E. coli* isolates, the dominant sequence type was ST131 (40.0%, 18/45), followed by ST648(8.9%,4/45), ST410(6.7%,3/45), ST10 and ST7358(n=2 for each). In total, 21 different sequence types were identified in the 45 KPC-positive *E. coli* strains, whereas other sequence types were identified in one strain including ST167, ST216, ST131* and so on.

Genetic environment analysis of *bla*$_{KPC}$ gene in *E. coli* revealed that 57.8% (26/45) of *bla*$_{KPC}$ gene was linked to Tn*4401*-associated structure IS*Kpn6*-*bla*$_{KPC}$-IS*Kpn7* while *bla*$_{KPC}$-IS*Kpn27* were observed in 15 strains (Figure S1 and Table S4).

Table 1 Antimicrobial Susceptibilities of Clinical Isolated *Escherichia Coli* Determined by the Broth Microdilution Method

Antimicrobial Agents	MIC (μg/mL)			Number (%) of Isolates			
	Range	MIC50	MIC90	Susceptible	SDD	Intermediate	Resistance
Amikacin	<2->256	4	16	264(62.3)	–	109(25.7)	51(12.0)
Aztreonam	<0.125->32	16	>32	166(39.2)	–	32(7.5)	226(53.3)
Cefotaxime	<0.125->64	16	32	118(27.8)	–	0	306(72.2)
Cefoxitin	2->64	8	>64	243(57.3)	–	46(10.9)	135(31.8)
Ceftazidime	<0.25->32	>32	>32	158(37.3)	–	17(4.0)	249(58.7)
Cefoperazone-sulbactam[a]	<0.06->512	16/4	128/4	235(55.4)	–	76(17.9)	113(26.7)
Cefepime	0.25->64	8	64	166(39.2)	64(15.0)	–	194(45.8)
Ciprofloxacin	<0.03->4	>4	>4	65(15.3)	–	9(2.1)	350(82.6)
Imipenem	<0.25->32	<0.25	1	382(90.1)	–	2(0.5)	40(9.4)
Meropenem	<0.25->64	<0.25	4	374(88.2)	–	7(1.7)	43(10.1)
Piperacillin	<0.125->256	>256	>256	48(11.3)	9(2.1)	–	367(86.6)
Piperacillin-tazobactam[b]	<0.125->256	4	>256	301(71)	18(4.2)	–	105(24.8)
Polymyxin B	<0.25–2	<0.25	0.5	424(100)	–	–	0
Tigecycline	<0.015–32	1	4	374(88.2)	–	29(6.8)	21(5.0)
Trimethoprim-sulfamethoxazole[c]	<0.125->16	4	>16	199(46.9)	–	–	225(53.1)

Notes: [a]For cefoperazone-sulbactam, only the concentration of cefoperazone was listed; [b]For piperacillin-tazobactam, only the concentration of piperacillin was listed; [c]For trimethoprim-sulfamethoxazole, only the concentration of trimethoprim was listed.
Abbreviation: SDD, susceptible-dose dependent.

Plasmids Typing

Among the 45 KPC-positive *E. coli*, most bla_{KPC} genes were found on the plasmids, except for *E. coli* strain 3385 and HS3555, which was located on the chromosome. The plasmids were IncC (n = 2), IncFIA and IncP1 (n = 1 each) among the four clinical isolates, whereas IncN was the most common plasmid type (11/40, 27.5%), followed by IncC, IncR, and IncFIB (n=3 each) in KPC-producing *E. coli* from the GenBank database, as shown in Figure 1.

Among the bla_{KPC}-producing *K. pneumoniae*, 379 strains carried only one KPC-harboring plasmid while 5 strains carried only one bla_{KPC} located on the chromosome (Table S5). Six strains were observed with two bla_{KPC} genes located on plasmid and the chromosome while 23 strains carried two KPC-positive plasmids. IncFII was the dominant plasmid type (43.3%,190/439), followed by IncFIB (11.6%,51/439), IncR (10.7,47/439), and repB (6.2%,27/439) as shown in Figure 2.

Phylogenomic Analysis

A core-genome alignment and maximum likelihood (SNP)-based phylogeny was obtained for all 114 *E. coli* isolates with ST131 and ST131* genomes which revealed a 3-clade structure identical to those previously described (Figure 3). ST131 clade A contains the previously-sequenced ERR161235 strain.[13] ST131 clade B containing previously-sequenced ERR161305 strain was very similar to clade C. ST131 clade C strains make up 59% (85/114) of the ST131 strains. Most of the bla_{KPC}-producing ST131 *E. coli* (89.5%,17/19) belonged to clade C ST131 lineage except for Ecol_AZ159(Accession number GCF_002012145.1) and Ecol_244(Accession number GCF_002012305.1) which belonged to clade B ST131 lineage. Two bla_{KPC}-postive clinical isolated *E. coli* HS1496 and HS2039 were in clade C ST131 lineage.

Antimicrobial Susceptibility Testing for Common Antibiotics in Clinical Isolated *E. Coli* and Its bla_{KPC}-Harboring Conjugants

The *E. coli* clinical isolates and their bla_{KPC}-harboring conjugants exhibited resistance to multiple β-lactams, including piperacillin-tazobactam, meropenem, imipenem, and ceftolozane-tazobactam, but susceptibility to ceftazidime-avibactam and cefepime-zidebactam. The MICs of cefepime-tazobactam ranged from 4 to 16 mg/L in bla_{KPC}-carriers, in contrast to the increased susceptibility when cefepime was in combination with another β-lactamase inhibitor, namely cefepime-zidebactam (0.125 to 1 mg/L). All the isolates were susceptible to tigecycline and polymyxin B (Table 2).

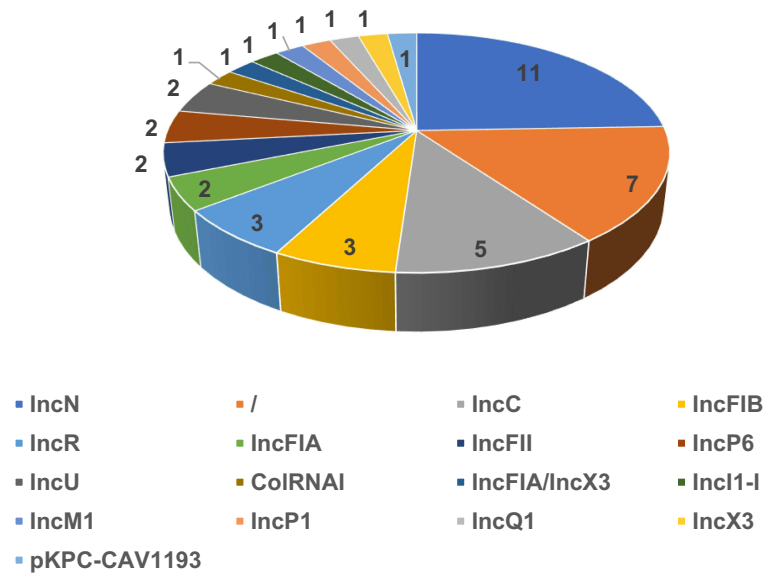

Figure 1 Plasmid typing composition of *Klebsiella pneumoniae* carbapenemase-producing *Escherichia coli* strains.

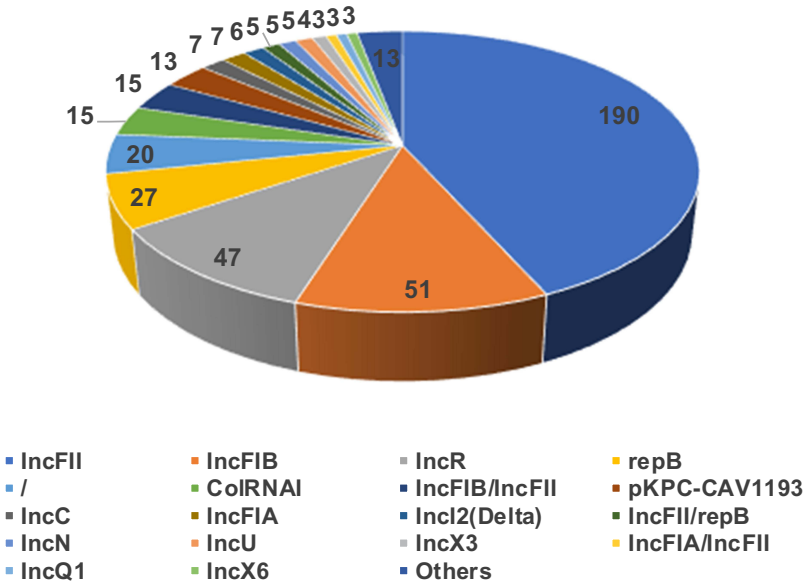

Figure 2 Plasmid typing composition of *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* strains.

Discussion

Antimicrobial resistance (AMR) has been identified as a major health threat and anticipated to cause 10 million deaths annually by 2050.[14] The World Health Organization (WHO) listed Carbapenem-Resistant Enterobacteriaceae (CRE) as critical pathogens and the highest prioritization of pathogens due to increasing antibiotic resistance and significant threat to humans.[15] Successful expansion of resistance clonal groups and frequent horizontal gene transfer (HGT) of carbapenemases harboring plasmids are causing increasing carbapenem resistance.[16]

Globally distributed in many bacterial genera, certain carbapenemases are associated with specific bacterial species and clonal groups. The international spread of KPC-producing *K. pneumoniae* has been linked to clonal complex 258 (CC258) while bla_{NDM} was the most prevalent carbapenemase gene in *E. coli*. Studies on bla_{KPC}-harboring *E. coli* is limited in China. Interestingly, research in Singapore showed that bla_{KPC} was the predominant carbapenemase type in

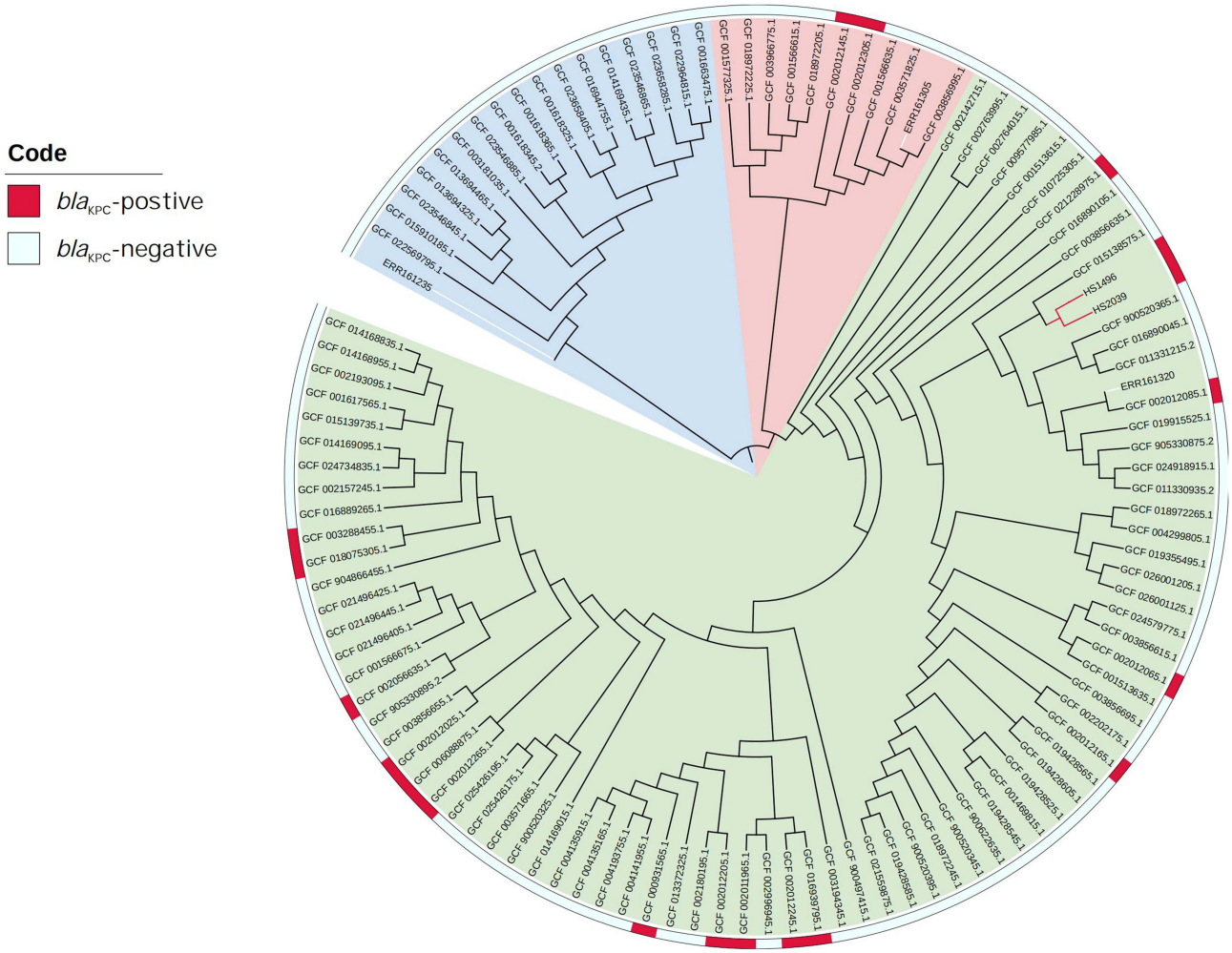

Figure 3 Maximum likelihood nonrecombinant core single nucleotide polymorphism (SNP)-based phylogeny of ST131 and ST131**Escherichia coli* (n = 114) from a clinical collection (n = 2) and the NCBI database (n = 112). The tree is rooted at the midpoint. Concentric ring indicates the presence (red code) of *bla*$_{KPC}$.
Note: ST131*(*adk* gene had 99.8% identity) indicate nearest ST.

E. coli whereas the prevalence of *bla*$_{KPC}$ was less than 3% in this study. The distribution of KPC-producing *E. coli* in our study, consistent with previous studies, was significantly different from that in Singapore, which may be related to geographic differences. ST131 was the dominant sequence type among the KPC-producing *E. coli* isolates in our study. Similar to the international spread of ST258 and its related variants in *K. pneumoniae*, ST131, the predominant *E. coli* lineage among extraintestinal *E. coli* isolates, was commonly reported to produce extended-spectrum β-lactamases and resistant to fluoroquinolones.[17] It is noteworthy that the prevalence of *E. coli* ST131 among KPC-producing *E. coli* strains demonstrated greater dissemination potential, making it possible to become the predominant carbapenemase type in China in the future.

Transmission of the KPC carbapenemase can be mediated by the mobility of transposons, horizontal transfer of plasmids, and clonal spread.[18] Bacteria and archaea possess defense systems such as CRISPR-Cas and restriction-modification (R-M) systems to protect microbes from infection by phages and other invading DNA such as plasmids. The CRISPR-Cas system in *K. pneumoniae* could effectively perturb the transfer of KPC plasmids, and the scarcity of the CRISPR-Cas system is a potential factor leading to the propagation of high-risk AMR linkages in *K. pneumoniae* CC258.[19,20] Interestingly, in *E. coli*, CRISPR-Cas systems are unable to target *bla*$_{KPC}$-producing plasmids and instead, it appears that the type I R-M system could impede *bla*$_{KPC}$-carrying plasmid conjugation in *E. coli*. The absence of the type I R-M system in ST131 contributed to the dissemination of *bla*$_{KPC}$ plasmids in this clonal group.[21]

Table 2 Susceptibility of *bla*$_{KPC}$-Positive Clinical Isolated *Escherichia Coli*, Their Conjugants and Recipient to Antimicrobial Agents

Isolates	β-Lactamase	IMP	MEM	FPT	FPZ	CZT	CZA	TZP[a]	SXT[b]	TGC	AMK	POL	CIP
HS0631	*bla*$_{CTX-M-15}$; *bla*$_{KPC-2}$;	128	>64	16	1	64	0.5	256	<0.25	0.125	<1	0.5	>8
C-HS0631	*bla*$_{KPC-2}$;	4	4	16	0.25	16	0.25	256	<0.25	0.25	<1	0.25	<0.06
HS1496	*bla*$_{OXA-1}$; *bla*$_{CTX-M-15}$; *bla*$_{TEM}$; *bla*$_{KPC-2}$	4	8	4	0.125	8	0.5	>256	>32	0.25	>128	0.25	>8
C- HS1496	*bla*$_{TEM}$; *bla*$_{KPC-2}$;	4	8	8	0.25	16	1	>256	>32	0.5	>128	0.25	>8
HS2039	*bla*$_{OXA-1}$; *bla*$_{CTX-M-15}$; *bla*$_{TEM}$; *bla*$_{KPC}$;	4	8	8	0.25	16	0.5	>256	>32	0.5	>128	1	>8
C-HS2039	*bla*$_{TEM}$; *bla*$_{KPC}$;	16	16	16	0.25	64	1	>256	<0.25	0.25	4	0.5	<0.06
HS2960	*bla*$_{CTX-M-14}$; *bla*$_{KPC-2}$;	16	32	16	0.5	32	1	>256	>32	0.125	2	0.25	>8
C-HS2960	*bla*$_{KPC-2}$;	8	8	4	0.25	64	0.25	>256	<0.25	0.125	<1	0.5	<0.06
HS3555	*bla*$_{CTX-M-15}$; *bla*$_{KPC-2}$;	4	16	8	0.125	8	0.5	128	<0.25	0.125	<1	0.5	>8
E. coli J53	/	0.25	≤0.03	≤0.03	0.06	0.5	0.25	4	<0.25	0.125	≤1	0.25	≤0.06

Notes: [a]For piperacillin-tazobactam, only the concentration of piperacillin was listed; [b]For trimethoprim-sulfamethoxazole, only the concentration of trimethoprim was listed. The original strains were shown in white bars, whereas the J53 conjugants were shown in gray.

Abbreviations: IPM, Imipenem; MEM, Meropenem; FPT, Cefepime-Tazobactam; FPZ, Cefepime-zidebactam; CZT, Ceftolozane-Tazobactam; CZA, ceftazidime-avibactam; TZP, Piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole; TGC, Tigecycline; AMK, Amikacin; POL, Polymyxin B; CIP, Ciprofloxacin; C, Conjugants of clinical isolates.

The global dissemination of KPC-producing *K. pneumoniae* CC258 is closely related to the epidemic IncF plasmids, contributing to the success of the high-risk linkage CC258–IncF, while IncN was the dominant plasmid type in KPC-producing *E. coli*. IncN plasmids are important drivers for the transmission of *bla*$_{KPC}$ between multiple bacterial species co-colonizing individual patients and environmental surfaces in several genomic studies.[22–24] Compared with IncF plasmids, *bla*$_{KPC}$-harboring IncN plasmids were more frequently identified in multiple bacteria from the same patients, demonstrating that IncN plasmids contributed to the inter-genera dissemination of the *bla*$_{KPC}$ genes. Interestingly, anti-restriction proteins encoded by *ardA* and *ardB* were identified in all or almost all sublineages of IncN plasmids, which may further facilitate their dissemination by inhibiting the function of the R-M system.[25]

This study collected *E. coli* isolates in 2017, and more strains isolated during different periods will be included to dynamically monitor the prevalence of *bla*$_{KPC}$ in *E. coli*. Novel defense systems, such as the BREX system, have been discovered against phages.[26] Further studies will be performed to determine whether these novel defense systems could block *bla*$_{KPC}$ plasmid acquisition in *E. coli*.

Conclusion

The detection rate of *bla*$_{KPC}$ is lower in *E. coli* compared with *K. pneumoniae. The* dominant sequence and plasmid types of *bla*$_{KPC}$-producing isolates differed between *E. coli* and *K. pneumoniae*. Further studies about the role of the defense system in acquisition of KPC-plasmids in *E. coli* will be performed to provide new insights into the low prevalence of *bla*$_{KPC}$.

Ethics Approval

Clinically isolated *E. coli* was obtained from the biological sample and strain bank of the Institute of Antibiotics, Huashan Hospital, Shanghai, China. The ethics committee of Huashan Hospital approved this study. This study would not do harm to rights, benefits, or health of the participants.

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Disclosure

The authors report no conflicts of interest in this work.

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