Infection and Drug Resistance

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ORIGINAL RESEARCH

Molecular Characteristics of *Escherichia coli* Causing Bloodstream Infections During 2010–2015 in a Tertiary Hospital, Shanghai, China

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Background: The bloodstream infections (BSI) caused by *Escherichia coli* pose a serious threat to human health. To explore molecular characteristics of *E. coli* causing BSI, we collected *E. coli* isolates causing BSI in Huashan Hospital, Shanghai, China during 2010–2015.

Methods: In all *E. coli* isolates causing BSI collected from this study, polymerase chain reaction (PCR) was used to detect ESBLs and carbapenemase genes, and minimum inhibitory concentrations (MICs) were determined with agar dilution method. Outer membrane proteins were examined by SDS-PAGE in carbapenem-resistant strains. The genetic background of bla_{KPC} gene was investigated by combining next-generation sequencing with a PCR mapping approach. Conjugation and transformation experiments were performed to verify the mobilization of bla_{KPC}. The transcription levels of the bla_{KPC} gene were measured by RT-PCR.

Results: During 2010–2015, a total of 207 *E. coli* BSI strains were isolated. The positive rates of β-lactamase resistant genes were 0.48% (bla_{KPC}), 57% (bla_{TEM}), 23.67% ($bla_{CTX\text{-}M\text{-}1}$), 18.84% ($bla_{CTX\text{-}M\text{-}9}$), and 1.93% ($bla_{SHV}$). High rates of bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, and $bla_{CTX\text{-}M\text{-}9}$ were consistent with the poor activity of third-generation cephalosporins and aztreonam in vitro, except for carbapenem and β-lactamase inhibitor combinations. Low susceptibility rates were observed for piperacillin (25.1%) in contrast to the increased susceptibility when combined with β-lactamase inhibitors, namely piperacillin-tazobactam (90.8%). Only one KPC-producing *E. coli* strain was detected. Despite the combination of OmpC loss, the low expression level of KPC may be responsible for its lower resistance to carbapenems compared to *E. coli* DH5α (pKP12-100).

Conclusion: *E. coli* strains isolated from BSI were still highly susceptible to carbapenems and β-lactamase inhibitor combinations, and $bla_{CTX\text{-}M}$ was the dominant genotype of ESBLs. The low expression of bla_{KPC} may be the reason for the low resistance to carbapenems.

Keywords: *Escherichia coli*, bloodstream infections, resistance mechanism, ESBLs

Introduction

Escherichia coli, a gram-negative, motile, facultative anaerobic, rod-shaped bacterium, is one of the most common hospital-acquired pathogens which could cause urinary tract infections, abdominal infections, bloodstream infections (BSI), etc.[1] Bacteremia represents a major cause of death with large increases in incidence and mortality.[2] *E. coli* is a leading cause of bloodstream infection, it ranks first as a cause of community-acquired episodes and second as a cause of hospital-acquired BSI in different world regions.[3] In addition, the incidence of *E. coli* BSI is increasing with associated high morbidity and mortality.[4] In a study from

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England, all-cause mortality rate in individuals with *E. coli* bacteremia was 18.2%.[5] And in China, one study showed that in 45 episodes of *E. coli* bacteremia, the 30-day all-cause mortality was 22.2%.[6]

β-lactams are commonly used in the treatment of BSI caused by *E. coli*. β-lactamase production remains the most important contributing factor to β-lactam resistance.[7] Extended-spectrum β lactamases (ESBLs), one group of β lactamases, have the ability to hydrolyze and cause resistance to various types of the β-lactam antibiotics, including the third-generation cephalosporins and monobactams except the cephamycins and carbapenems.[8,9] The most common ESBLs belong to three groups: TEM, SHV, and CTX-M types.[8] The CTX-M β lactamases, now exceeding 50 different types, can be divided into five groups based on their amino acid identities: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25.[10] Since their first description, class A extended-spectrum β-lactamases (ESBLs) producing *E. coli* continue to thwart our best clinical efforts. ESBLs-producing *E. coli* remains an important reason for therapy failure with cephalosporins and have serious consequences for infection control.[7]

Based on data from a multicenter randomized controlled trial, carbapenem is recommended as first-line treatment for infections outside of the urinary tract caused by ESBLs-producing *E. coli*.[11] In *E. coli*, carbapenem resistance is typically caused by two main mechanisms: production of carbapenemases and β-lactamase activity combined with structural mutations.[12] When combined with the mutation of outer membrane proteins or drug efflux pumps, ESBLs and AmpC are capable of conferring carbapenem resistance.[12] According to the Ambler classification method, carbapenemases are members of the molecular class A, B, and D beta-lactamases. Class A and D enzymes have a serine-based hydrolytic mechanism, while class B enzymes are metallo-beta-lactamases that contain zinc in the active site.[13,14] Of these, the KPC carbapenemases are the most prevalent, found mostly on plasmids in *Klebsiella pneumoniae*.[15]

Given the increasing importance and the fact that BSI caused by ESBLs-producing *E. coli* are an increasing therapeutic challenge, we investigated the molecular characteristics and antimicrobial susceptibility profiles of BSI caused by *E. coli* during 2010–2015 in Huashan Hospital, Shanghai, China.

Materials and Methods

Sources of Strains

A total of 207 non-duplicate *E. coli* isolates were collected from blood cultures of the inpatients of Huashan Hospital, Fudan University from 2010 to 2015. *E. coli* was identified using the Vitek 2 system. *E. coli* A49, selected from 207 strains mentioned previously, was used as the positive reference for outer membrane proteins with complete OmpC, OmpF, and OmpA. Plasmid pKP12-100 (Supplementary Data 1) was extracted from KPC-producing *K. pneumoniae* KP100-12 isolated from Huashan Hospital, not belonging to the 207 strains mentioned previously. This plasmid was used for transformation.

MIC Determination

In 207 *E. coli* strains in this study (Supplementary Data 2), minimum inhibitory concentrations (MICs) of cefotaxime, cefepime, ceftazidime, cefoxitin, ampicillin, aztreonam, piperacillin, piperacillin-tazobactam, meropenem, imipenem, ertapenem, fosfomycin, ciprofloxacin, amikacin, and gentamicin were determined with agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as routine controls for agents mentioned previously. The criterion of the susceptibility of fosfomycin was based on EUCAST (European Committee on Antimicrobial Susceptibility Testing) (Resistance standard: MIC ≥ 32μg/mL). We investigated the effect of efflux pump inhibitors cyanide 3-chlorophenylhydrazone (CCCP) on the carbapenems' susceptibility in the carbapenem-resistant *E. coli*. The concentration of CCCP was 25μg/mL.

Detection of Resistance Genes

Polymerase chain reaction (PCR) was used to detect β-lactmase such as bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}25}$, bla_{SHV} and the carbapenemases genes such as bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{SPM}, bla_{AIM}, bla_{VIM}, bla_{OXA}, bla_{GIM}, bla_{BIC}, bla_{SIM}, bla_{DIM}.[16] And in our laboratory, we possessed isolates which were used as positive controls for the following genes: bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{OXA}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$, bla_{SHV} and bla_{TEM}. Amplification was carried out as follows: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30s, 60°C for 30s and 72°C for 1 min; and a final elongation step at 72°C for 7 min. Primers were listed in Supplementary Data 3.

Analysis of Outer Membrane Proteins (OMPs) of KPC-Producing Strain

Briefly, the suspension was sonicated on ice for about 10 minutes (60 cycles for 5 seconds with 5-second intervals). The cell extracts were centrifuged at 15,600 *g* 4°C for 60 minutes, then we removed the supernatant and added 200 μL 1×PBS, 25 μL10% Sarcosyl to resolve the protein. We repeated the procedure and suspended the OMP with 80 μL 1×PBS. Porins were loaded onto 15% SDS-polyacrylamide gel. After a 150-min electrophoresis of 80 V, the membrane was stained with 0.1% Coomassie brilliant blue (Beyotime, China).[17]

Conjugation and Transformation Experiments

Conjugation and transformation experiments were performed to verify the transferability of bla_{KPC}. Plasmid pKP12-100 was extracted from a bla_{KPC}-positive isolate through phenol-chloroform method and then transformed into the recipient strain *E. coli* DH5α. *E. coli* J53, an azide-resistant strain was used for conjugation experiments.[18] Agar plate containing ampicillin (50μg/mL) was used to screen for transformants. Conjugation strain was selected on LB agar plates supplemented with 50μg/mL ampicillin and 150μg/mL sodium azide. PCR with primers Kpc-RT (listed in Table 1) and sequencing were used to verify transformants and conjugation strain.

Genetic Environment of bla_{KPC} Positive Strain

DNA was extracted from EC-A59 (TIANamp Bacteria DNA Kit) and next-generation sequencing was performed (Supplementary Data 4). Flanking sequences of bla_{KPC} were extracted from the contig harboring bla_{KPC} and analyzed by Blastn. The genetic background of bla_{KPC} gene in *E. coli* A59 obtained from this study was investigated by combining next-generation sequencing with a PCR mapping approach with the primers listed in Table 1. We obtained the genetic background of bla_{KPC} gene in *E. coli* A59 for further visualized genetic environment comparisons of bla_{KPC}-positive strains with Easyfig.

Table 1 Primers Presented Below Were Used for RT-qPCR of bla_{KPC} Gene in This Study

Primer	Direction	Sequence (5′→3′)
mdh	Forward Reverse	TGGCAAACTGAAACGGATA ACGGCTGGATTGATGAAC
Kpc-RT	Forward Reverse	GAACCTGCGGAGTGTATG TGTGCTTGTCATCCTTGTT
Kpc-UP	Forward Reverse	TGGCAAACTGAAACGGATA ACGGCTGGATTGATGAAC

Reverse Transcription-Quantitative PCR

Total RNAs from clinical isolates were extracted using TaKaRa MiniBEST Universal RNA Extraction Kit and cDNA synthesis was performed with PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). The transcription levels of the bla_{KPC} gene were measured with FastStart Universal SYBR Green Master (ROX)(Roche) as recommended by the manufacturers. The *mdh* housekeeping gene was used as the internal reference. Primer sequences are listed in Table 1.

Results

Molecular Characteristics of 207 *E. coli* Isolates Causing BSI

During 2010 to 2015, a total of 207 *E. coli* isolates causing BSI were collected. The overall *E. coli* isolates causing BSI is on the rise, especially in 2015 (Figure 1). The antibiotic resistance rates were listed as follows: 67.1% (cefotaxime), 36.2% (cefepime), 42.5% (ceftazidime), 24.2% (cefoxitin), 87.4% (ampicillin), 45.9% (aztreonam), 59.9% (piperacillin), 5.8% (piperacillin-tazobactam), 0% (meropenem) and 0% (imipenem), 5.3% (ertapenem), 19.8% (fosfomycin), 76.8% (ciprofloxacin), 9.2% (amikacin), 61.4% (gentamicin) (Table 2). Low susceptibility rates were observed for piperacillin (25.1%) in contrast to increased susceptibility when combined with β-lactamase inhibitors, namely piperacillin-tazobactam (90.8%).

The positive rates of β-lactamase resistant genes were 0.48% (bla_{KPC}), 57% (bla_{TEM}), 23.67% ($bla_{CTX\text{-}M\text{-}1}$), 18.84% ($bla_{CTX\text{-}M\text{-}9}$), 1.93% ($bla_{SHV}$). The rates of bla_{ESBLs} by year were shown in Figure 2. The most common bla_{ESBLs} was $bla_{CTX\text{-}M\text{-}1}$, followed by $bla_{CTX\text{-}M\text{-}9}$. Only one *E. coli* strain A59 was discovered harboring $bla_{KPC\text{-}2}$ gene. Genes $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}25}$ and bla_{NDM}, bla_{IMP}, bla_{SPM},

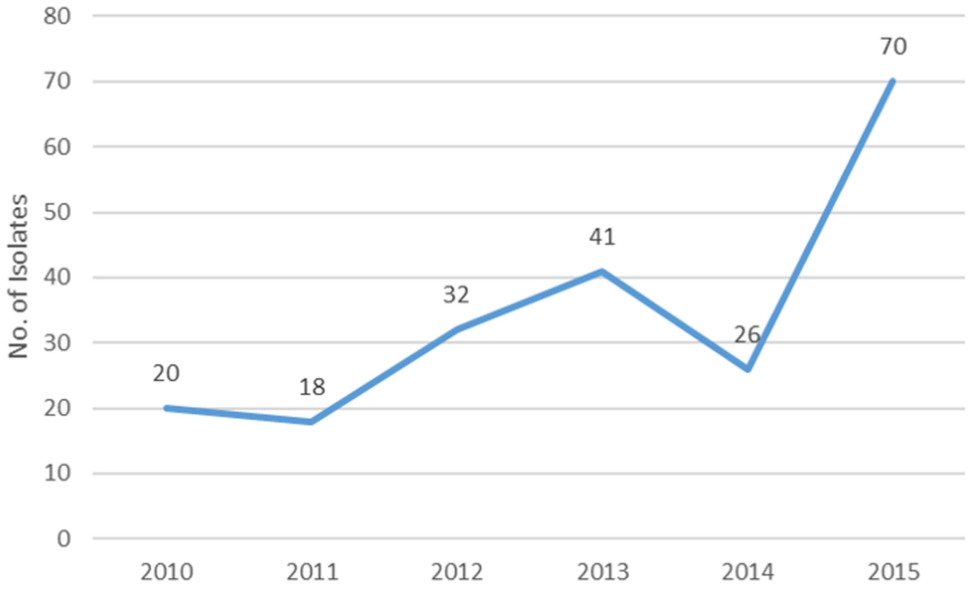

Figure 1 The isolation numbers of *Escherichia coli* causing bloodstream infections according to the year.

bla_{AIM}, bla_{VIM}, bla_{OXA}, bla_{GIM}, bla_{BIC}, bla_{SIM}, bla_{DIM} were not detected in this study.

OMP Profile of bla_{KPC}-Positive Strain

SDS-PAGE analysis revealed different OMP profiles among the two isolates (Figure 3). OmpC loss was observed in isolate *E. coli* A59 compared to *E. coli* A49.

Conjugation and Transformation Experiments of the bla_{KPC} Positive Strain

The bla_{KPC-2}-carrying plasmid named pKP12-100 was extracted from *K. pneumoniae* 12–100 and transformed into *E. coli* DH5α. The MIC of the transformant *E. coli* DH5α (pKP12-100) can be seen in Table 3. Conjugation and transformation of *E. coli* A59 were failed with at least three repeats.

Table 2 Antimicrobial Susceptibility of *Escherichia coli*

Antimicrobial Agents	Breakpoint (ug/mL)			MIC (ug/mL)			Number (%) of Isolates		
	S	I	R	Range	MIC50	MIC90	R	I	S
Amikacin	≤16	32	≥64	2~>128	4	16	19 (9.2)	0	188(90.8)
Gentamicin	≤4	8	≥16	0.5~>128	64	>128	127 (61.4)	2(0.97)	78(37.7)
Cefotaxime	≤1	2	≥4	<0.06~>128	64	>128	139 (67.1)	2(0.97)	66(31.9)
Fosfomycin	<32	–	≥32	0.25~>128	1	>128	41 (19.8)	—	166(80.2)
Cefepime	≤2	4–8 (SDD)	≥16	<0.06~>128	4	128	75 (36.2)	43(20.8)	89(43.0)
Ceftazidime	≤4	8	≥16	0.125~>128	4	128	88 (42.5)	11(5.3)	108(52.2)
Cefoxitin	≤8	16	≥32	1~>128	8	128	50 (24.2)	18(8.7)	139(67.1)
Ciprofloxacin	≤0.25	0.5	≥1	<0.06~>128	16	128	159(76.8)	16(7.7)	32(15.5)
Ampicillin	≤8	16	≥32	2~>128	>128	>128	181(87.4)	2(0.97)	24(11.6)
Piperacillin	≤16	32–64	≥128	1~>128	128	>128	124(59.9)	31(14.98)	52(25.1)
Piperacillin-Tazobactam	≤16/4	32/4-64/4	≥128/4	1~>128	2	6	12(5.8)	7(3.4)	188(90.8)
Aztreonam	≤4	8	≥16	0.125->128	8	128	95(45.9)	13(6.3)	99(47.8)
Meropenem	≤1	2	≥4	<0.06~1	<0.06	0.25	0	0	207(100)
Imipenem	≤1	2	≥4	<0.06~2	0.125	0.5	0	1(0.48)	206(99.5)
Ertapenem	≤ 0.5	1	≥2	<0.06~32	<0.06	0.5	11(5.3)	3(1.5)	193(93.2)

Note: In the combinations, the concentration of tazobactam was 4 mg/L constant.
Abbreviations: MIC, minimal inhibitory concentration; S, susceptible; I, intermediate; R, resistant.

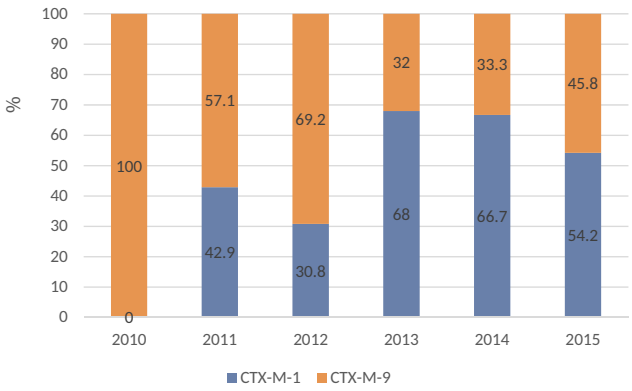

Figure 2 The distribution of ESBLs genes in ESBLs-producing *Escherichia coli*.

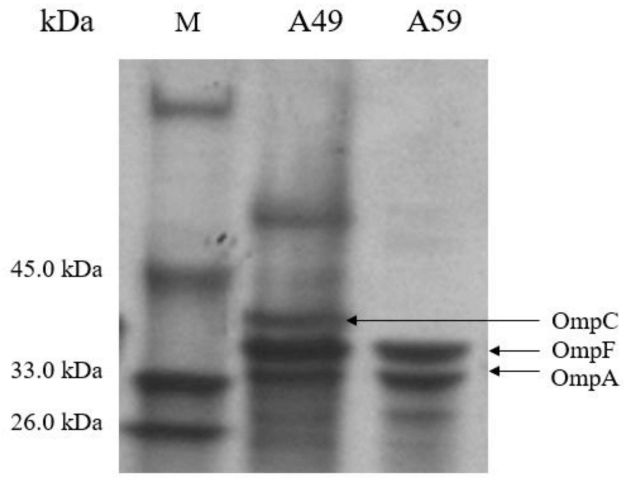

Figure 3 OMP profiles of *E. coli* A59 compared to its parental strain, *E. coli* A49. OMPs were profiled by SDS-PAGE. Lane 1, marker; lane 2, *E. coli* A49 (control strain); lane 3, *E coli* A59.The horizontal arrows on the right indicate the positions of OMPs: OmpC, OmpF, and OmpA.

Carbapenems' Susceptibility and Efflux Pump Inhibition Test of bla_{KPC} Positive Strains

The MICs of KP12-100 to imipenem, meropenem, and ertapenem were 128μg/mL, 128μg/mL and ≥256μg/mL respectively while the transformant of KP12-100 was 8μg/mL, 4μg/mL and 128μg/mL. The MICs of *E. coli* A59 to imipenem, meropenem, and ertapenem were 2μg/mL, 1μg/mL and 16μg/mL respectively. Efflux pump inhibitor carbonyl cyanide 3-chlorophenyl-hydrazone resulted in at least 8-fold decrease in the MIC of imipenem, meropenem and ertapenem for *E. coli* DH5α (pKP12-100). And an 8-fold decrease in the MIC of ertapenem was observed in DH5α-P12-100 (Table 3).

Genetic Environment of bla_{KPC}-Positive Strain

Combining next-generation sequencing with a PCR mapping approach, genetic environment of bla_{KPC} in *E. coli* A59 was IS*Kpn6*-bla_{KPC-2}-IS*Kpn27*-IS*26* sharing the same core structure as that from the chromosome of ECO3385 (CP029420.1). Since bla_{KPC-2} was not able to be transferred to the recipient we supposed that the bla_{KPC} in *E. coli* A59 may be located on the chromosome (Figure 4).

RT-qPCR of bla_{KPC} Gene

Compared with the imipenem resistant control strain, *E. coli* DH5α (pKP12-100), the transcription levels of

Table 3 EC-A59 Was an Ertapenem-Resistant *E. coli* Strain with OmpC Loss Isolated from This Study. KP12-100 Was a KPC-Producing *K. pneumoniae* KP100-12 Isolated from Huashan Hospital. DH5α-P12-100 Was Constructed in This Study. Through the Results of Efflux Pump Inhibition Among the Three Isolates Mentioned Above Were Listed Below. Remarkable MIC Changes of EC-A59 and DH5α-P12-100 Were Observed in Ertapenem

MIC (ug/mL) Strains	IMP	IMP+CCCP	MEM	MEM+CCCP	ETP	ETP+CCCP
EC-A59	2	0.25	1	0.5	16	2
DH5α-P12-100	8	1	4	0.5	128	0.5
KP12-100	128	64	128	128	≥256	≥256

Abbreviations: IMP, imipenem; MEM, meropenem; ETP, ertapenem; CCCP, carbonyl cyanide-m-chlorophenylhydrazone.

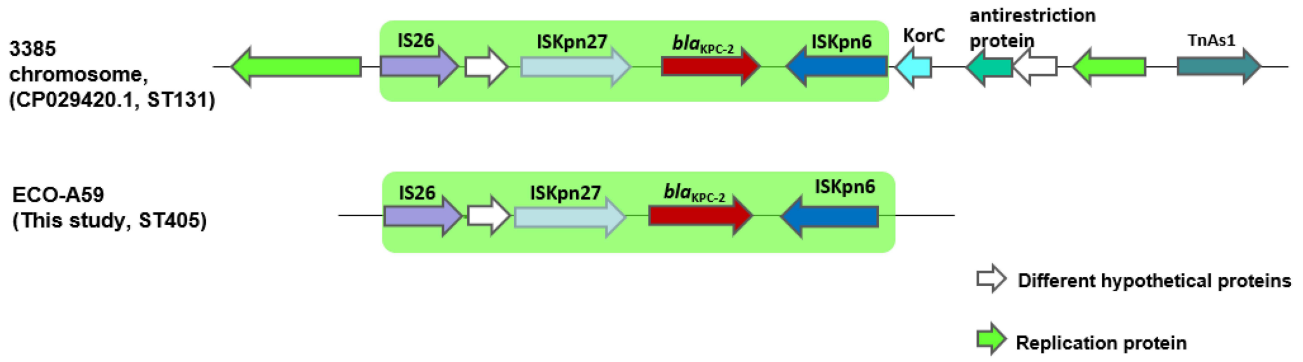

Figure 4 Comparisons between the structures of ECO3385 and EC-A59. IS*Kpn6*-bla_{KPC-2}-IS*Kpn*27-IS*26* is shown in green.

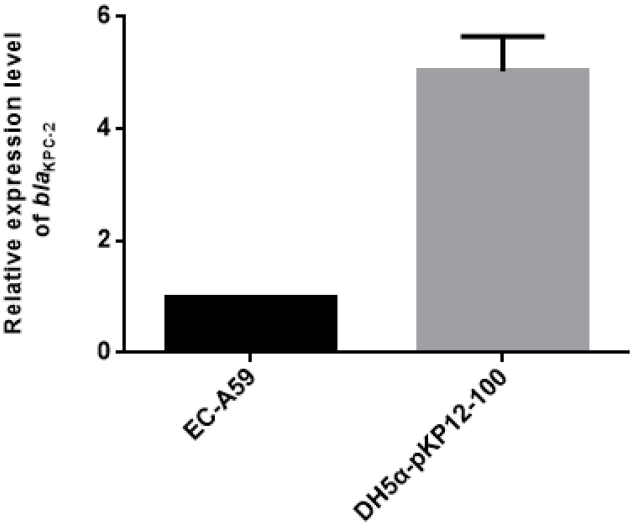

Figure 5 Transcription level of bla_{KPC-2}. Compared with DH5α-pKP12-100, the imipenem-resistant control strain, the transcription levels of bla_{KPC-2} were five-fold lower in isolate *E. coli* A59 (EC-A59).

bla_{KPC} gene were five-fold lower for isolate *E. coli* A59 (Figure 5). At least 280-bp sequence upstream of the bla_{KPCs} in the two strain was identical, see Supplementary Data 5, indicating that they shared the same promoter region of bla_{KPC}.

Discussion

Data from the CHINET Antimicrobial Surveillance Program showed that the proportion of *E. coli* in BSI pathogens is 22.2%, which is a leading cause of BSI in China. The antimicrobial resistance rates of *E. coli* isolated from 35 hospitals in 2017 throughout China were as follows: 25.2% (cefepime), 25.2% (ceftazidime), 12.2% (cefoxitin), 86.5% (ampicillin), 4.1% (piperacillin-tazobactam), 1.5% (meropenem) and 1.5% (imipenem), 2% (ertapenem), 5.2% (fosfomycin), 57.8% (ciprofloxacin), 2.3% (amikacin) (http://www.chinets.com/). In our research, the antimicrobial resistance rates were almost consistent with these data. Although all isolates were susceptible to meropenem and imipenem, there were 11 *E. coli* isolates resistant to ertapenem. According to the existing research, the expression of β-lactamases such as an AmpC β-lactamase or an ESBL combined with porin loss participated in ertapenem resistance in *Enterobacteriaceae* isolates.[19]

ESBLs are often encoded by plasmids that are transferable from strain to strain and between bacterial species.[20,21]

In our study, bla_{CTX-M} was the dominant genotype among the ESBLs-producing *E. coli* which is consistent with the situation in China.[22] The occurrence of ESBLs is increasing.[8] Data from rural Thailand showed very high rates, reaching 69.3% in 2010. The great majority of CTX-M alleles identified in Thailand belonged to group 9.[23]

Carbapenem-resistant *E. coli* is posing great challenges to human health.[12,24] The plasmid-mediated horizontal transmission of carbapenemase genes is the main cause of the surge in the prevalence of CRE. NDM, one of the metallo-β-lactamases, is the predominant carbapenemase in *E. coli* while KPC carbapenemases are the most prevalent ones among class A carbapenemase group and found mostly on plasmids in *K. pneumoniae*.[15,25] *K. pneumoniae* are the predominant carriers of bla_{KPC}, mainly associated with the clonal group 258 (CG258) including ST258, ST11, ST340, ST512, and others.[26,27] One study indicated that type I-E CRISPR-Cas system targeting the backbone regions of bla_{KPC}-bearing IncF plasmids influences the acquisition of bla_{KPC} plasmid in *K. pneumoniae*. The absence of type I-E CRISPR-Cas in CG258 contributes to the dissemination of IncF epidemic resistance plasmids in this clonal complex.[28] Until now, reports about KPC-producing *E. coli* have been rare and

the low detection rate of *bla*$_{KPC}$ in *E. coli* remains obscure.

From the results of the national surveillance of CRE strains in China, it was shown that the core structure of IS*Kpn6*-*bla*$_{KPC-2}$-IS*Kpn27* was conservative in KPC-producing *K. pneumoniae* and *E. coli* strains.[29] In this study only the chromosomes of ECO3385 (CP029420.1) and *E. coli* A59 shared the same core structure. In addition to the conservative sequences, they still hold another transposable element IS*26*, and this kind of structure was a little bit different from the previously reported pK048 (IncFIIK5) harboring non-Tn*4401* elements in China.[30] Whether certain divergences between *K. pneumoniae* and *E. coli* resulted in the intergeneric diversity of transposable genetic elements should be taken into consideration.

Previous research showed that KPC enzymes contribute to the carbapenem resistance in *K. pneumoniae*.[26] Compared with the KP12-100, the MIC of carbapenem for its transformation strain *E. coli* DH5α (pK12-100) had decreased at least 16-fold, which indicated the existence of other resistance mechanisms. *E. coli* A59 was a *bla*$_{KPC}$ positive strain with OmpC loss which was not resistant to meropenem and imipenem. Considering the low expression level and the failed conjugation and transformation experiments, we propose that the decreased MIC of meropenem and imipenem may be due to the low expression level of *bla*$_{KPC}$ located on the chromosome of *E. coli* A59. Only one KPC-producing *E. coli* was detected in this study. More strains will be included to clarify the overall detection rate of *bla*$_{KPC}$ in *E. coli* in the future.

Ethical Statement

The strains we used in this study were obtained from the biological sample and strains bank of the Institute of Antibiotics, Huashan Hospital, Shanghai, China. They came from normal clinical testing and were stored in the strains bank. The ethics committee of Huashan Hospital authorized our study and written informed consent was not required. This study would not do harm to rights, benefits, and health of the subjects, and the privacy and personal identity information of the subjects will not be included in this study.

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Disclosure

The authors report no conflicts of interest in this work.

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