

Novel genetic environment of the plasmid-mediated KPC-3 gene detected in *Escherichia coli* and *Citrobacter freundii* isolates from China

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Received: 1 September 2010 / Accepted: 20 November 2010 / Published online: 14 December 2010
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Abstract The imipenem and meropenem-resistant strains *Citrobacter freundii* HS70 and *Escherichia coli* HS510 were isolated from patients in Shanghai, China. By isoelectric focusing, PCR amplification and sequencing, these strains were each found to produce four β -lactamases: TEM-1, KPC-3, SHV-7 and CTX-M-14. A conjugation experiment and plasmid restriction digestion revealed that the *bla*_{KPC-3} gene was located on the same plasmid in both isolates. Bidirectional primer walking sequencing showed that the nucleotide sequence surrounding the 3.8 kb *bla*_{KPC-3} contained a 671-bp insertion similar to that previously characterized in China. The insertion was located between the promoter and the coding region of the *bla*_{KPC-3} gene. Susceptibility testing performed on recombinant strains carrying the *bla*_{KPC-3} gene with or without the insertion revealed that minimum inhibitory concentrations of imipenem, meropenem, cefepime, and cefotaxime for *E. coli* EMU-KPC3 (without insertion) were four times higher than that of *E. coli* EKPC3 (with insertion). The 671 bp insertion reduced *bla*_{KPC-3} expression significantly. Taken together, these results suggest that KPC-3-producing *C. freundii* and *E. coli* have begun to emerge in our hospital.

Introduction

The extensive use of carbapenems has resulted in the emergence of carbapenem-resistant *Enterobacteriaceae* strains [4]. The resistance may be mediated by the production of carbapenemases [15, 19], as well as AmpC hyper-production combined with decreased outer membrane permeability due to loss or alteration of porins [14]. The Ambler class A *Klebsiella pneumoniae* carbapenemase (KPC) enzymes [13] are able to hydrolyze all known β -lactam-containing molecules and are the most frequently observed class A carbapenemases.

KPC-1, a plasmid encoded β -lactamase, was first identified from *K. pneumoniae* in North Carolina (USA) and is identical to the *bla*_{KPC-2} gene by sequencing [25, 26]. Strains harboring *bla*_{KPC-1/KPC-2} have also been isolated from patients in France [9], Israel [12], South America [18], Greece [3], and China [20]. The *bla*_{KPC-3} sequence (GenBank AM774409) is found in the same genetic environment as *bla*_{KPC-2} in *Salmonella cubana* 4707 (GenBank AF481906) [5], and this KPC enzyme is now prevalent in America [22], Israel [6], and the United Kingdom [23].

In this study, we identified strains of *Citrobacter freundii* and *E. coli* isolated from Chinese patients that express KPC-3. The background of the *bla*_{KPC-3} gene was different from that reported outside of China and it was similar to that reported in China previously [27].

Materials and methods

Bacterial strains and plasmids

The imipenem and meropenem-resistant strain *C. freundii* HS70 was isolated from the urine of a 53-year-old female

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patient hospitalized in Huashan Hospital, Fudan University. The imipenem and meropenem-resistant strain *E. coli* HS510 was isolated from urine of a 59-year-old female inpatient at the same hospital. Both strains were identified by Vitek-32 (BioMerieux, Marcy, France). *E. coli* J53 was used as a recipient in conjugal mating experiments, whereas *E. coli* DH5 α was used for cloning. Other derivative strains and plasmids used in this study are listed in Table 1.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for organisms were determined by the Mueller-Hinton (M-H) agar dilution method according to guidelines of the Clinical and Laboratory Standards Institute [2]. Antimicrobial agents evaluated included imipenem, meropenem, cefepime, cefotaxime, ampicillin, ciprofloxacin, and gentamicin. All were obtained from Oxoid (Basingstoke, England). *E. coli* ATCC25922 was used for quality control.

Conjugation experiments and plasmid restriction enzyme digestion analysis

Transfer of imipenem resistance was studied by performing conjugation experiments as previously described [24] with *E. coli* J53 as the recipient. Transconjugants were selected from agar plates supplemented with sodium azide (100 μ g/mL; Oxoid, Basingstoke, England) and ceftazidime (2 μ g/mL; Oxoid, Basingstoke, England), and identified by VITEK-32. For the plasmid restriction enzyme analysis, *Xba*I and *Cl*aI

(Takara, Dalian, China) were used. Digested plasmid DNA samples from transconjugants were then analyzed by electrophoresis on 0.6% agarose gels at a constant voltage of 100 V for 0.5 h.

Isoelectric focusing of β -lactamases

Crude cell lysates were prepared by a previously described freeze–thaw procedure [17]. Isoelectric focusing was performed as described by Matthew and Harris [8]. Cell extracts were loaded onto prepared polyacrylamide gel plates (pH 3 to 9; Amersham Biosciences, Uppsala, Sweden) and electrophoresed to equilibrium using Pharmacia PhastSystem (Uppsala, Sweden). β -lactamases were then visualized by staining the gel with a 0.05% solution of nitrocefin (BD Biosciences, San Jose, CA, USA). The isoelectric points of TEM-1, KPC-3, SHV-7 and CTX-M-14 were determined by comparison to known pIs of the β -lactamases (TEM-12, pI 5.25; TEM-28, pI 6.1; SHV-7, pI 7.6; and ACT-1, pI 9.0).

PCR analysis and nucleotide sequencing

Crude genomic DNA was extracted from the isolates by heat lysis. β -lactamase genes were identified by PCR with specific primers designed to sequences of known β -lactamase genes, including *bla*_{TEM}, *bla*_{SHV}, *bla*_{KPC}, *bla*_{CTX-M-1}, *bla*_{CTX-M-9}, and *bla*_{CTX-M-2}. For PCRs the *LA Taq* DNA polymerase (Takara, Dalian, China) was used according to the manufacturer's instructions. Primer sequences are listed in Table 2.

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Reference
Plasmids		
pACYC184	Cloning vector	
pacyc184-KPC3	Insertion of 727-2902 region between <i>Bam</i> H I and <i>Eco</i> RI restriction site in pACYC184	FJ609231
pMU-acyc184-KPC3	Deletion of 1188-1706 in pacyc184-KPC3	FJ609231
pHS70	Plasmid from <i>E. coli</i> J 53 (pHS70)	This study
pHS510	Plasmid from <i>E. coli</i> J 53 (pHS510)	This study
Strains		
<i>Citrobacter freundii</i>		
HS70	Clinical isolate	This study
<i>E. coli</i>		
HS510	Clinical isolate	This study
<i>E. coli</i> J 53 (pHS70)	<i>E. coli</i> J53 transconjugant derived from HS70	This study
<i>E. coli</i> J 53 (pHS510)	<i>E. coli</i> J53 transconjugant derived from HS510	This study
EKPC3	<i>E. coli</i> DH5a containing pacyc184-KPC3	This study
EMU-KPC3	<i>E. coli</i> DH5a containing pMU-acyc184-KPC3	This study
DH5 α	<i>E. coli</i> reference lab strain	
J53	<i>E. coli</i> reference lab strain	
ATCC25922	<i>E. coli</i> reference lab strain	

Table 2 Primers for PCR amplification of the β -lactamases genes and for cloning

ESBL or plasmid	Primer	Sequence	Position	GenBank accession number or reference
KPC	KPC-F	5'-ATGTCAGTGTATCGCCGTCT-3'	131-150	AF297554
	KPC-R	5'-TTTTTCAGAGCCTTACTGCCC-3'	1023-1042	
TEM	TEM-F	5'-ATAAAATTCTTGAAGAC-3'	1-17	X54604.1
	TEM-R	5'-TTACCAATGCTTAATCA-3'	1075-1059	
SHV	SHV-F	5'-TGGTTATGCGTTATATTCGCC-3	69-89	X98100.1
	SHV-R	5'-GCTTAGCGTTGCCAGTGCT-3'	936-918	
CTX-M-1	M1F	5'-GGTTAAAAAATCACTGCGTC-3	65-84	X92506
	M1 R	5'-TTGGTGACGATTTTAGCCGC-3	928-909	
CTX-M-9	M9F	5'-ATGGTGACAAAAGAGAGTGCA-3	1-20	AF252621.2
	M9R	5'-CCCTTCGGCGATGATTCTC-3'	870-852	
	M2F	5'-ATGATGACTCAGAGCATTCG-3'	304-323	
CTX-M-2	M2R	5'-TGGGTTACGATTTTCGCCGC-3'	1169-1150	AJ416343.1
pacyc184-KPC3	KPC-3 F	5'-GCCTGGTCCGAATTCCTCGTCATCCGCAGACCAAC-3'	727-747	FJ609231
	KPC-3R	5'-GCCTGGTCCGGATCCCGCGCAGACTCCTAGCCTAAA-3'	2882-2902	
pMU-acyc184-KPC3	MU-KPC-3 F	5'-CTAACGTGAGTTTTCGTCCACTGAGCG-3'	1816-1844	FJ609231
	MU-KPC-3R	5'-AAGTCATTTTCAATATTATTGAAGCATTT ATC-3'	1112-1144	

ESBL extended-spectrum beta-lactamase

PCR amplifications were performed and PCR products were then sequenced by an ABI 3730 analyzer, and the obtained sequences were aligned with sequence data from GenBank.

Analysis of the genetic environment of the *bla*_{KPC-3} gene and plasmid construction

The genetic context of the *bla*_{KPC-3} gene was examined by bidirectional primer walking sequencing, performed on plasmids from transconjugants using primers designed previously [20]. The obtained sequence was aligned with β -lactamase sequences from the GenBank database using the BLAST program. Primers KPC-3 F and KPC-3R were used to amplify the DNA fragments, which included the entire *bla*_{KPC} gene and the region flanking the insertion in the plasmid isolated from the Chinese patients. The obtained PCR products and plasmid pACYC184 were digested using *Bam* HI and *Eco*RI, then ligated using T4 DNA ligase. The obtained plasmid pACYC184-KPC3 was electroporated into *E. coli* DH5 α . Clones were selected on Luria-Bertani agar plates containing chloramphenicol (40 mg/mL) and imipenem (1.5 mg/mL). Primers MU-KPC-3F and MU-KPC-3R were designed to amplify the whole recombinant plasmid pACYC184-KPC3 without the 671 bp insertion fragment. The obtained PCR products were ligated by using a MutantBEST kit (Takara) to produce the plasmid pMU-ACYC184-KPC3. The recombinant plasmids pACYC184-KPC3 (with insertion) and pMU-ACYC184-KPC3 (without insertion) were then indi-

vidually transformed into *E. coli* DH5 α by the calcium phosphate method.

Results

Antimicrobial resistance

E. coli HS510 was isolated from the urine of a 59-year-old female patient and was found resistant to imipenem and meropenem, with a MIC of 16 μ g/mL for both antibiotics. *C. freundii* HS70 was isolated from the urine of a 53-year-old female patient and had a MIC of \geq 128 μ g/mL for both antibiotics (Table 3).

Isoelectric focusing and PCR analysis of β -lactamases

In order to understand the antimicrobial resistant phenotype, β -lactamases were analyzed by isoelectric focusing, PCR and PCR product sequencing. Isoelectric focusing of clinical isolates revealed that both *E. coli* HS510 and *C. freundii* HS70 produced four β -lactamases which possess pIs of 5.4, 6.7, 7.6, and 8.1 (Fig. 1), respectively. To determine which lactamases they were, PCR was performed on DNA from the clinical isolates with primers specific for KPC, TEM, SHV, CTX-M-1, CTX-M-2, and CTX-M-9. Sequencing analysis of two clinical isolates confirmed that both *E. coli* HS510 and *C. freundii* HS70 carried *bla*_{TEM-1}, *bla*_{CTX-M-14}, *bla*_{SHV-7} and *bla*_{KPC-3}.

Table 3 Antimicrobial susceptibility patterns^a

Antimicrobial agent(s)	MIC ($\mu\text{g/mL}$)						
	HS70 ^b	<i>E. coli</i> J 53(pHS70)	HS510	<i>E. coli</i> J 53(pHS510)	EKPC3	EMU-KPC3	25922 ^c
Imipenem	≥ 128	2	16	2	8	32	≤ 0.0625
Meropenem	≥ 128	2	16	2	8	32	≤ 0.0625
Cefepime	≥ 128	8	64	8	16	64	≤ 0.0625
Cefotaxime	≥ 128	16	≥ 128	16	16	64	≤ 0.0625
Ampicillin	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	2
Ciprofloxacin	≥ 128	≤ 0.0625	≥ 128	≤ 0.0625	≤ 0.0625	≤ 0.0625	≤ 0.0625
Gentamicin	≥ 128	≤ 0.0625	≥ 128	≤ 0.0625	≤ 0.0625	≤ 0.0625	≤ 0.0625

^a Minimum inhibitory concentrations (MICs) were determined by the M-H agar dilution method according to guidelines of the Clinical and Laboratory Standards Institute

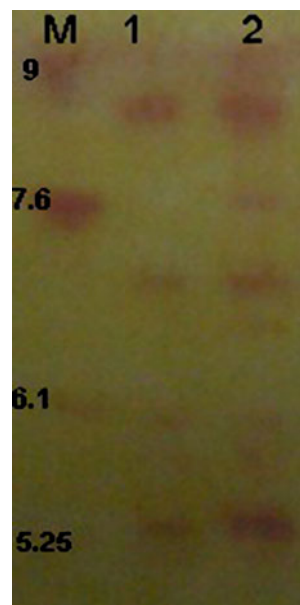
^b Clinical isolates: *E. coli* HS510 and *C. freundii* HS70. Transconjugants: *E. coli* J 53 (pHS510); *E. coli* J 53 (pHS70). Recombinants: EKPC3, *E. coli* DH5a/ pacyc184-KPC3; EMU-KPC3, *E. coli* DH5a/ pMU-acyc184-KPC3. Lab reference *E. coli* strain: 25922

^c *E. coli* ATCC 25922 was used as quality control of antimicrobial susceptibility testing

Plasmid profile analysis

Conjugation experiments successfully transferred a plasmid from both clinical isolates to the recipient *E. coli* J53. In order to understand which resistant genes can be transferred by plasmid conjugation, we used PCR to identify every β -lactamase gene that the two clinical isolates contained on transconjugants. PCR results confirmed that both transconjugants only carried the *bla*_{KPC-3} gene. Restriction enzyme (*Xba*I and *Cla*I) digestion of plasmids from transconjugants showed that they had identical enzyme digestion maps (Fig. 2). Our results indicated that the *bla*_{KPC} gene was located on the same plasmid in both clinical isolates.

Fig. 1 Isoelectric focusing patterns of clinical isolates *E. coli* HS510 and *C. freundii* HS70. Lane 1: Cell lysate prepared from *C. freundii* HS70 producing TEM-1 (pI 5.4), KPC-3 (pI 6.7), SHV-12 (pI 7.6), and CTX-M-14 (pI 7.9). Lane 2: Cell lysate prepared from *E. coli* HS510 producing TEM-1, KPC-3, SHV-12 and CTX-M-14



Characterization of the genetic environment of the *bla*_{KPC-3} gene

Bidirectional primer walking performed on transconjugated plasmids produced a 3,850 bp fragment. The nucleotide

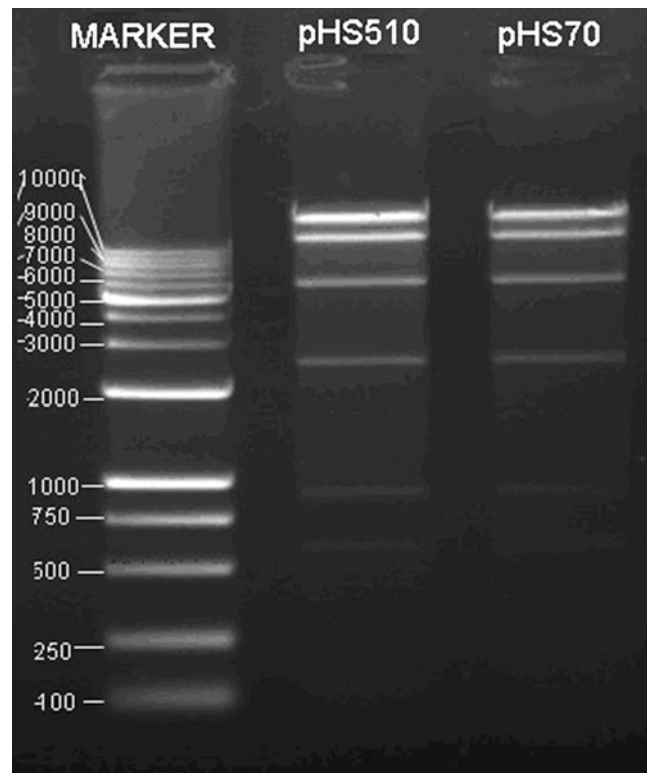


Fig. 2 Electrophoretic analysis of *Xba*I- and *Cla*I-digested plasmids. Lane 2: *E. coli* transconjugants of HS70. Lane 3: *E. coli* transconjugants of HS510. Marker lane: 100-bp and 1-Kb DNA ladders (Takara)

sequence was assigned GenBank accession number FJ609231. Sequence alignment showed that the FJ609231 sequence was similar to AM774409.1. FJ609231 carried the *bla*_{KPC-3} gene; EU176014.1 and FJ628167 each carried a *bla*_{KPC-2} gene. Furthermore, FJ609231 showed a similar structure and context with FJ628167, except for a 671-bp fragment insertion between the *bla*_{KPC-3} promoter and the *bla*_{KPC} coding region (between positions 20571 and 20572 of FJ628167; see Fig. 3). As for FJ628167, an ISKpn6-like element is located downstream of the *bla*_{KPC-3} gene and a Tn3 transposon and ISKpn8 is located upstream of the *bla*_{KPC-3} gene [27]. The additional 671 bp insertion looks like a piece of TEM1. The nucleotide sequence area is from 982 to 1928 of FJ609231, and looks like a rearranged FJ223605.1.

Antimicrobial susceptibility testing of recombinants

To investigate the effects of the inserted 671-bp fragment on *bla*_{KPC-3} expression, plasmids pKPC3-184 and pMU-KPC3-184 were constructed using the vector pACYC184. The plasmid pKPC3-184 was derived from plasmid pHS70, and pMU-KPC3-184 was derived from plasmid pKPC3-184. The genetic environment of the *bla*_{KPC} gene on pKPC3-184 was the same with pHS70. The genetic environment of the *bla*_{KPC} gene on pMU-KPC3-184 was the same with pHS70 except for a 671-bp fragment insertion between the *bla*_{KPC-3} promoter and the *bla*_{KPC} coding region. Recombinants *E. coli* EKPC3 and *E. coli* EMU-KPC3 (with plasmids pKPC3-184 and pMU-KPC3-184, respectively) were constructed and tested for antimicrobial susceptibility. The MICs of *E. coli* EMU-KPC3 for imipenem and meropenem were four times higher than that of *E. coli* EKPC3 (32 µg/mL vs. 8 µg/mL) as were MICs for cefepime and cefotaxime (64 µg/mL for *E. coli* EMU-

KPC3 vs. 16 µg/mL for *E. coli* EKPC3), implying that the 671-bp fragment insertion decreased KPC-3 expression.

Discussion

This is the first time that the *bla*_{KPC-3} gene was reported in China. Nine KPC variants have so far been described (KPC-2 to KPC-10; KPC-1 and KPC-2 are identical) in different parts of the world; variants of KPC-1/2 differ by, at most, two amino acid substitutions [1, 5, 6, 16, 21, 22]. The emergence of KPC-type carbapenem-hydrolyzing enzymes is alarming. *E. coli* transconjugants carrying the *bla*_{KPC-3} gene from either clinical isolate in our study had MICs of 2 µg/mL for both imipenem and meropenem. Additional mechanisms must underlie the drug resistance of clinical isolates *C. freundii* HS70 and *E. coli* HS510 since their MICs for imipenem and meropenem were greater than that of their *E. coli* transconjugants. Previous reports showed that the lost porins OmpK36 and OmpK35 are perhaps associated with the increase in MICs for carbapenems [22].

Our conjugation experiments, plasmid restriction enzyme digestion analysis and PCR of the β-lactamase gene confirmed that *bla*_{KPC-3} from both clinical isolates is located on the same conjugational plasmid. Sequence analysis revealed that 3.8 Kb of DNA sequence surrounding the *bla*_{KPC-3} gene of these two isolates was also the same, and its construction was similar to AM774409.1 from *Enterobacter cloacae* and EU176014.1 from *Klebsiella pneumoniae*. It was composed of ISKpn8, the *bla*_{KPC-3} promoter, a 671-bp insertion, the *bla*_{KPC-3} gene and an ISKpn6-like element (Fig. 3). This DNA fragment is one part of a Tn3-based transposon, Tn4401, which is likely to be the origin of *bla*_{KPC} mobilization and further insertion into various plasmids of non-clonally related organisms [10].

This report provides further confirmation of the putatively transposable element found on plasmids of *K. pneumoniae* and *P. aeruginosa* that may be responsible for the rapid global dissemination of this gene. The region upstream of the *bla*_{KPC-3} identified here differs from AM774409.1 and EU176014.1 (Fig. 3). A 671-bp fragment insertion was found between the *bla*_{KPC-3} promoter and the *bla*_{KPC-3} coding region (between positions 20571 and 20572 of FJ628167). This finding confirms Naas's proposition [10] that this region of the element is unstable, and suggests that other isoforms of Tn4401 do exist. Naas et al. identified a 100-bp deletion upstream of *bla*_{KPC} from two clinical strains of *K. pneumoniae*, namely, GR and YC [10].

Interestingly, we found that the insertion decreased *bla*_{KPC-3} expression. Our results provide important information on the genetic environment of *bla*_{KPC-3} genes identified in clinical isolates in China and the significance of *bla*_{KPC-3} in this genetic context warrants further study.

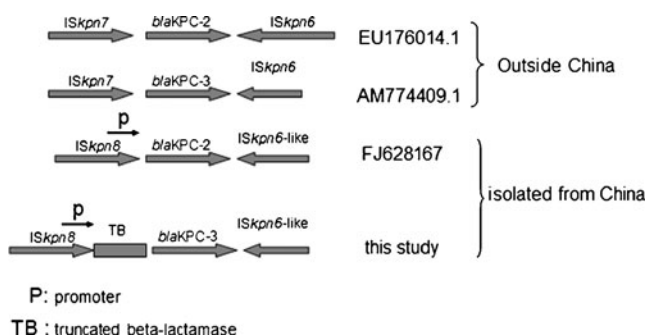


Fig. 3 Schematic representation of novel genetic structure involved in *bla*_{KPC-3} gene found in bacteria isolated from Chinese patients. EU176014.1 contains ISKpn7, *bla*_{KPC-3} and ISKpn6; AM774409.1 contains ISKpn7, *bla*_{KPC-2} and ISKpn6; FJ628167 contains ISKpn8, *bla*_{KPC-2} and an ISKpn6-like element; this study contained ISKpn8, truncated β-lactamase, *bla*_{KPC-3} and an ISKpn6-like element

Funding The present study was supported in part by the Key Technologies Research and Development Program for Infectious Diseases of China (no. 2009ZX10004-104), the National Natural Science Foundation of China (no. 81071396), funding from the Shanghai Medical Key Discipline and the Science Foundation of Shanghai Jinshan (2008-3-5).

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