Characterization of the \textit{bla}_{KPC-2} and \textit{bla}_{KPC-3} genes and the novel \textit{bla}_{KPC-15} gene in Klebsiella pneumoniae

Dongguo Wang,¹ Wei Hou,² Jiayu Chen,³ Yonghua Mou,⁴ Linjun Yang,⁵ Liqin Yang,⁶ Xiulian Sun⁷ and Meiyun Chen⁸

¹Department of Clinical Laboratory Medicine, Taizhou Municipal Hospital and Institute of Molecular Diagnostics, Taizhou University, Taizhou, Zhejiang, PR China
²Department of Infectious Diseases, Taizhou Municipal Hospital, Taizhou University, Taizhou, Zhejiang, PR China
³Department of Laboratory Medicine, Medical School of Taizhou University and Institute of Molecular Diagnostics, Taizhou University, Taizhou, Zhejiang, PR China
⁴Department of Hepatobiliary Surgery, Taizhou Municipal Hospital, Taizhou University, Taizhou, Zhejiang, PR China
⁵Department of Thyroid and Breast Surgery, Taizhou Municipal Hospital, Taizhou University, Taizhou, Zhejiang, PR China
⁶Department of Orthopaedics, Taizhou Municipal Hospital, Taizhou University, Taizhou, Zhejiang, PR China
⁷Emergency Department, Taizhou Municipal Hospital, Taizhou University, Taizhou, Zhejiang, PR China
⁸Department of Paediatrics, Taizhou Municipal Hospital, Taizhou University, Taizhou, Zhejiang, PR China

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Three \textit{Klebsiella pneumoniae} isolates exhibiting high-level resistance to carbapenem were analysed by PCR, PFGE, gene mapping, plasmid conjugation and Southern blot hybridization using a \textit{bla}_{KPC} probe. In addition to the frequently reported \textit{bla}_{KPC-2} and \textit{bla}_{KPC-3} genes, a novel \textit{bla}_{KPC-15} gene was identified in one of the isolates. The results of plasmid analysis and Southern blot hybridization revealed that the three \textit{bla}_{KPC} genes were located on transferable plasmids exhibiting three different patterns. The patterns A, B and C were observed in the genetic makeup of each individual plasmid, and all three structures contained \text{IS}^{Kpn6}\text{-like and IS}^{Kpn8}\text{transposons. The results of the gene mapping and hybridization experiments performed with the \textit{bla}_{KPC} probe demonstrated that the plasmids harboured the three genes at approximately the 85.0, 54.0 and 73.0 kb positions. The study concluded that carbapenem resistance in the three isolates was primarily due to the production of carbapenem-hydrolysing \beta-lactamase.

INTRODUCTION

\textit{Klebsiella pneumoniae} carbapenemase (KPC), a member of the Ambler’s molecular class A, subgroup 2f enzymes (Bush \textit{et al.}, 1995), hydrolyses all \textit{\&\-lactams, including penicillins, cephalosporins, monobactams and carbapenems, and is weakly inhibited by clavulanic acid and tazobactam

\textit{Klebsiella pneumoniae} are often isolated from patients with carbapenem-resistant Enterobacteriaceae (CRE) infections (Nordmann \textit{et al.}, 2009; Queenan & Bush, 2007). KPC was first described as \textit{bla}_{KPC-4} (afterwards, it was determined to be identical to \textit{bla}_{KPC-2}) and was detected on a transferable plasmid in a \textit{K. pneumoniae} isolate from a hospital in North Carolina in 2001 (Yigit \textit{et al.}, 2001a, b). \textit{bla}_{KPC-2} was also discovered in \textit{Klebsiella} spp. (Smith Moland \textit{et al.}, 2003; Yigit \textit{et al.}, 2003) and \textit{Salmonella enterica} (Mirigliano \textit{et al.}, 2003), and subsequently \textit{bla}_{KPC-3} and \textit{bla}_{KPC-4} have been described (Palepou \textit{et al.}, 2005; Woodford \textit{et al.}, 2004). KPCs were also identified in many types of \textit{Enterobacteriaceae} (Paterson, 2006) and have been discovered in Europe, Asia and America (Gootz \textit{et al.}, 2009; Hawser \textit{et al.}, 2011; Queenan & Bush, 2007; Robledo \textit{et al.}, 2010; Roth \textit{et al.}, 2011).

\textsuperscript{t}These authors contributed equally to the manuscript.

Abbreviations: KPC, \textit{Klebsiella pneumoniae} carbapenemase; ICU, intensive care unit; IS, insertion sequence.

The GenBank/EMBL/DDBJ accession number for the \textit{bla}_{KPC-15} variant gene sequence of \textit{Klebsiella pneumoniae} is KC433553.
Of all KPCs, KPC-2 and KPC-3 were the most frequently reported variants (Kitchel et al., 2009; Robledo et al., 2010). To date, the KPC enzymes KPC-2 to KPC-15 have been observed, and KPC-15 is the most recently discovered enzyme in the study. Tn4401 was identified earlier in nonclonally related KPC-producing K. pneumoniae and Pseudomonas aeruginosa isolates and indicated a frequent and dynamic process of transposition. Tn4401 possesses the resolvase tspR, the bla_{KPC} gene, and two insertion sequences (ISs), ISKpn6 and ISKpn7 (Naas et al., 2008). Based on the above information, this article focuses on the structural and environmental features of genetic variants of the bla_{KPC-2}, bla_{KPC-3} and the novel bla_{KPC-15} genes.

**METHODS**

**Bacterial isolates.** The three isolates of *K. pneumoniae*, Kp1433, Kp1658 and Kp1241, were isolated from patients’ sputum and blood specimens and demonstrated resistance to carbapenem antibiotics (imipenem and meropenem). In this study, three isolates and demonstrated resistance to carbapenem antibiotics. The isolates were obtained from the Hepatobiliary Surgery Department in June 2011. The isolate Kp1241 was obtained from the Hepatobiliary Surgery Department in June 2012 (Fig. 1). The isolates were identified as *K. pneumoniae* using a Vitek GNI card (bioMérieux), and the strain names were determined by sequence analysis of the 16–28S rRNA genes (Midi Laboratories).

**Antimicrobial susceptibility testing.** The values of MIC from 18 antimicrobial agents, including imipenem (ertapenem), meropenem, cephalosporin, aminoglycosides, ampicillin/sulbactam, piperacillin/tazobactam and cefoperazone/sulbactam, were determined through a broth microdilution method using a Microscan plate reader (Microscan). *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC 27853 were used as controls. The results were interpreted according to standard methods (CLSI, 2012).

**PFGE.** Sucrose blood agar plates (Amresco, X-Y Biotech, Shanghai, China) were inoculated and incubated for 18 h at 35 °C. Bacterial growth was harvested and suspended in 2 ml sterile saline to an optical density of 0.5 at 550 nm. DNA was prepared as described by Woodford et al. (2004). Electrophoresis was performed at 6 V cm⁻¹ with an initial switch time of 1 s to a final switch time of 40 s at 14 °C in 0.5 × Tris/borate/EDTA (TBE) buffer for 16 h. The gel was stained with ethidium bromide (1 μg ml⁻¹) for 20 min and was destained in distilled water for 20 min. DNA profiles were analysed by visual examination, as described by Tenover et al. (1995).

**PCR amplification and gene mapping.** Plasmids from the three *K. pneumoniae* isolates were obtained using an AxyPrep Plasmid Miniprep kit (Axygen Biosciences). Before using the kit, 10 μl RNase A was added to Buffer S1. LB medium was used to incubate 1–4 ml of bacterial suspension for 18 h at 35 °C; the medium was then collected and centrifuged at 12 000 g for 1 min to pellet the bacteria. The supernatant was decanted to the greatest extent possible, and the bacterial pellet was resuspended in 250 μl Buffer S1 by vortexing. Next, 250 μl of Buffer S2 and 350 μl of Buffer S3 were added and mixed gently, and the manufacturer’s instructions for plasmid extraction were subsequently followed. To elute the purified plasmid DNA, 60 μl of eluent was added to the centre of the membrane, allowed to stand for 1 min at room temperature, and then centrifuged at 12 000 g for 1 min. The supernatant was discarded, and the plasmids harbouring bla_{KPC-2}, bla_{KPC-3}, bla_{KPC-15} and the neighbouring genes were isolated.

PCR was used to amplify bla_{KPC-2}, bla_{KPC-3}, bla_{KPC-15} and the neighbouring genes (bla\_TEM, istA, istB, tspR) in the plasmids of *K. pneumoniae* using the primers listed in Table 1. PCR was also performed for bla_{KPC-2}, bla_{KPC-3}, bla_{KPC-15} and the neighbouring genes in *E. coli* J53 Az² (resistance to sodium azide) using the following cycling conditions: 3 min at 94 °C and 30 cycles of 1 min each of 94 °C, 52 °C and 72 °C, followed by a final elongation step of 10 min at 72 °C. The total reaction volume was 25 μl, and the eluent was 10 μl. After PCR amplification, PCR products were separated by gel electrophoresis on a 0.6% agarose gel run at 90 V for 90 min in 0.5 X TBE buffer. The bands were cut out of the gel, and the plasmid DNA fragments of different sizes were recovered. These fragments served as templates for the amplification of the bla_{KPC-2}, bla_{KPC-3} and bla_{KPC-15} genes by PCR. The initial positions of bla_{KPC-2}, bla_{KPC-3} and bla_{KPC-15} were determined on the plasmids in reference to the estimated sizes of plasmid DNA (Wang et al., 2003). The bla_{KPC} map, including its flanking regions, was constructed and analysed for the presence of Tn4401 or other structures based on the sequencing results described previously.

**Conjugation and Southern blot hybridization.** Following a previously described protocol (Wang et al., 2003), bacterial conjugation experiments were performed in lysogeny broth (LB) using *E. coli* J53 Az² as the recipient and the three clinical isolates as the donors. Donor and recipient organisms were incubated in 4 ml LB overnight without shaking. The transconjugants were selected on tryptase soy agar (TSA; BD, Becton, Dickinson and Company, USA) plates containing sodium azide (300 mg l⁻¹) and imipenem (2 mg l⁻¹) by incubating for 18 to 24 h at 35 °C.

The DNA samples from the three clinical isolates and transconjugants were transferred to a Duralon-UV nylon membrane (Stratagene)
from the agarose gel by capillary action in 20 \times \text{SSC} transfer buffer (1 \times \text{SSC}=0.15 \text{M NaCl, 0.015 M sodium citrate}) overnight and were then fixed using a Stratalinker UV cross-linker (Stratagene) (Palepou et al., 2005). Membranes were hybridized with a 6-carboxy-fluorescein-labelled \textit{bla}\textsubscript{KPC} probe created using a fluorescence labelling kit (Stratagene) in accordance with the manufacturer's instructions. Hybridization was performed using the Illuminator chemiluminescent detection system (Stratagene) in accordance with the manufacturer's protocol.

**RESULTS**

**Antimicrobial susceptibility testing**

The MIC values for isolates Kp1433, Kp1658 and Kp1241 on 18 antimicrobial agents are shown in Table 2. The results indicated that all three of the isolates not only produced resistance to the antimicrobial agents but also exhibited high-level resistance to imipenem, meropenem, ertapenem (Fig. 1, Table 2), cephalosporin, aminoglycosides, ampicillin/sulbactam, piperacillin/tazobactam and ceftoperazone/sulbactam.

**PFGE, PCR amplification and DNA sequencing**

PFGE analysis of \textit{XbaI}-digested genomic DNA indicated that there were three distinct patterns, A, B and C, in the isolates (Fig. 1). The PCR results indicated that a band of approximately 1000 bp was obtained in the \textit{bla}\textsubscript{KPC-2} and \textit{bla}\textsubscript{KPC-3} reactions, and a band of approximately 1431 bp was obtained for \textit{bla}\textsubscript{KPC-15}, encompassing the entire KPC coding region. The sequences assembled with the ContigExpress program showed the genetic environment of \textit{bla}\textsubscript{KPC-2}, \textit{bla}\textsubscript{KPC-3} and \textit{bla}\textsubscript{KPC-15} in the three isolates. The allelic variants of \textit{bla}\textsubscript{KPC-3}, \textit{bla}\textsubscript{KPC-2} and novel \textit{bla}\textsubscript{KPC-15,} as well as \textit{bla}\textsubscript{TEM-1} and \textit{bla}\textsubscript{TEM-12}, were identified by sequence analysis.

**Conjugation experiments, gene mapping and plasmid analysis**

Plasmids of isolates Kp1433, Kp1658 and Kp1241 successfully transferred carbapenem resistance into \textit{E. coli} J53 AzR in conjugation experiments. Transconjugants exhibited resistance to imipenem, meropenem, ertapenem, cephalosporin, aminoglycosides, ampicillin/sulbactam and piperacillin/tazobactam, as well as ceftoperazone/sulbactam. The genetic map and plasmid analysis showed that isolate Kp1241 harboured a novel \textit{bla}\textsubscript{KPC-15} gene, \textit{bla}\textsubscript{TEM-12}, \textit{tnp}R and \textit{tnp}A genes or transposons, and Kp1241 harboured a novel \textit{bla}\textsubscript{KPC-15} gene, \textit{bla}\textsubscript{TEM-12}, \textit{tnp}R and \textit{tnp}A genes or transposons. The allelic variants of \textit{bla}\textsubscript{KPC-3}, \textit{bla}\textsubscript{KPC-2} and novel \textit{bla}\textsubscript{KPC-15} were identified by sequence analysis.

### Table 1. Primer sequences designed for amplification of the \textit{bla}\textsubscript{KPC} gene and the relevant genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Product size (bp)</th>
<th>Reference</th>
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For, forward; Rev, reverse.

<table>
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<th>Transconjugants</th>
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<td>4/8/4</td>
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<td>16/16/16</td>
<td>16/4/4</td>
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<td>128/128/128</td>
<td>64/64/32</td>
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<td>Tobramycin</td>
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<td>8/16/16</td>
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<td>8/4/4</td>
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<td>64/128/128</td>
<td>16/16/16</td>
</tr>
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<td>Cefotetan</td>
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<td>64/128/128</td>
<td>32/16/16</td>
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<td>256/256/256</td>
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<td>Cefoperazone/sulbactam</td>
<td>0.0125</td>
<td>64/128/128</td>
<td>32/16/16</td>
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</tbody>
</table>

novel \( \text{bla}_{\text{KPC-15}} \) variants were located on plasmids approximately 85.0, 54.0 and 73.0 kb in length, respectively (Fig. 2).

Genetic structural analysis of \( \text{bla}_{\text{KPC-2}}, \text{bla}_{\text{KPC-3}} \) and \( \text{bla}_{\text{KPC-15}} \)

Following DNA sequence analysis with the BLAST program (Altschul et al., 1990), three types of genetic structures, A, B and C, were observed. Structure A containing the \( \text{bla}_{\text{KPC-3}} \) gene had four upstream genes, \( \text{istA}, \text{istB}, \text{tnpA} \) (IS\(_{Kpn8}\)) and \( \text{tnpR} \), and a downstream \( \text{tnpA} \) gene (IS\(_{Kpn6}\)-like). This was almost identical to the genetic structure of the \( \text{bla}_{\text{KPC-2}} \) gene in GenBank accession no. JX500679 except that the \( \text{bla}_{\text{KPC-3}} \) gene was replaced by the \( \text{bla}_{\text{KPC-2}} \) gene (Fig. 3a) and was within the region of the Tn\(_{4401}\) transposon, but was of a different Tn\(_{4401}\) isotype that contained the \( \text{tnpR} \), \( \text{bla}_{\text{KPC-3}}, \text{istA} \) and \( \text{istB} \), IS\(_{Kpn6}\)-like and IS\(_{Kpn8}\) genes or transposons. Structure B contained the \( \text{bla}_{\text{KPC-2}} \) gene and had four upstream genes; \( \text{bla}_{\text{TEM-1}}, \text{tnpA} \) (IS\(_{Kpn8}\)), \( \text{tnpR} \) and the \( \text{tnpA} \) gene (IS\(_{Kpn6}\)-like) (Fig. 3b). Structure C was

![Fig. 2. Electrophoretogram of the \( \text{bla}_{\text{KPC}} \) gene with genetic mapping. (a) Plasmids of isolates Kp1433, Kp1658 and Kp1241 were separated using gel electrophoresis. Lane M, E. coli V517 standard (Fermentas, Thermo Fisher Scientific) plasmid size marker. Lanes 1–3, plasmids of isolates Kp1241, Kp1658 and Kp1433 detected in three different sizes. Lanes 4–6, plasmids of isolates Kp1241, Kp1658 and Kp1433 harbouring the \( \text{bla}_{\text{KPC-15}}, \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{KPC-3}} \) genes that were approximately 73.0, 85.0 and 54.0 kb in length, respectively; lanes 7 and 8, plasmids of isolate Kp1241 approximately 5.1 and 3.5 kb in length; lanes 9 and 10, plasmids of isolate Kp1658 approximately 5.1 and 3.5 kb in length; lanes 11 and 12, plasmids of isolate Kp1433 approximately 5.1 and 3.7 kb in length. (b) Southern blot with the \( \text{bla}_{\text{KPC}} \) probe for the three isolates. Lane 13, plasmid harbouring the approximately 54.0 kb \( \text{bla}_{\text{KPC-2}} \) gene; lane 14, plasmid harbouring the approximately 73.0 kb \( \text{bla}_{\text{KPC-15}} \) gene; lane 15, plasmid harbouring the approximately 85.0 kb \( \text{bla}_{\text{KPC-3}} \) gene. KPCs were identified using \( \text{bla}_{\text{KPC}} \) probes.](image-url)
In the current study, three clinical isolates were observed for genes in the plasmid of each isolate. The values of MICs were three plasmids of different lengths that contained the blaKPC genes. Three genetic patterns (A, B and C) were observed in the isolates using PFGE. The three isolates were unrelated, suggesting that the individual isolates with different structures had been determined based on varied sources, that is, either the KPC elements or the genetic variants of the isolates in the hospital. The plasticity of the K. pneumoniae genome allows the active evolution of variants to occur, particularly during heavy usage of antibiotics (Gootz et al., 2009).

**DISCUSSION**

It is well known that carbapenem resistance is due to the presence of carbapenemase and cephalosporinase combined with the loss of porin (Gaynes & Culver, 1992; Nordmann et al., 2009). In the USA, KPC is the most common mechanism for carbapenem resistance (Gupta et al., 2011; Nordmann et al., 2009; Yigit et al., 2001a, b). In the current study, three clinical isolates were observed to carry the blaKPC-2 and blaKPC-3 genes and the novel blaKPC-15 gene on plasmids of different lengths. As we observed in our results, KPCs can hydrolyse carbapenems, which results in resistance to imipenem, meropenem and ertapenem. Moreover, the results from our susceptibility testing also indicated resistance to cephalosporin, aminoglycosides, ampicillin/sulbactam, piperacillin/tazobactam and cefoperazone/sulbactam in the isolates. Such resistance may involve β-lactamase, aminoglycoside and quinolone genes in the plasmid of each isolate. The values of MICs for E. coli isolates J53 AzK, Kp1433, Kp1658, Kp1241 and transconjuncts confirmed that the blaKPC genes were present in plasmids observed by the conjugation experiment.

Three genetic patterns (A, B and C) were identified among the isolates using PFGE. The three isolates were unrelated, suggesting that the individual isolates with different structures had been determined based on varied sources, that is, either the KPC elements or the genetic variants of the isolates in the hospital. The plasticity of the K. pneumoniae genome allows the active evolution of variants to occur, particularly during heavy usage of antibiotics (Gootz et al., 2009).

The rapid spread of the blaKPC genes could partly be due to the genes’ mobility and to mutations of plasmids or transposons (Cai et al., 2008; Cuzon et al., 2008; Yigit et al., 2001a, b, 2003). Rasheed described blaKPC genes that were usually identified on large plasmids of similar size, while our gene mapping and analysis demonstrated that there were three plasmids of different lengths that contained blaKPC genes. The blaKPC-15, blaKPC-2 and blaKPC-3 genes were located on plasmids measuring approximately 73.0, 85.0 and 54.0 kb in length, respectively. These genes, together with the most widespread extended spectrum β-lactamase on the plasmids, were also responsible for aminoglycoside-induced resistance, as well as resistance to other antibiotics such as quinolones (qnrA, qnrB) (Cai et al., 2008; Monteiro et al., 2009; Yigit et al., 2003).

The schematic representation of the genetic variants of blaKPC-2, blaKPC-3 and blaKPC-15 indicated that structure A belongs to a region measuring approximately 10 kb in Tn4401 (Rasheed et al., 2008), and the blaKPC-3 gene was located on an approximately 54.0 kb plasmid. Genetic
structure C was identical to structure B except for the \( blak_{KPC} \) genes (Fig. 3). To the best of our knowledge, this report is the first to describe structure C with a novel \( blak_{KPC-15} \) variant and \( blatem-12 \) gene. These three genetic structures include putative IS\( Kpn8 \) and IS\( Kpn6 \)-like transposases, which possess two unrelated IS compositions. This finding suggests that the genes are capable of moving into different plasmids by the insertion of transposons.

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