KPC-2 carbapenemase and DHA-1 AmpC determinants carried on the same plasmid in Enterobacter aerogenes

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This study was conducted to analyse the presence of a plasmid-mediated carbapenem resistance mechanism in a clinical Enterobacter aerogenes isolate from a patient from Jiangsu province, People’s Republic of China. PCR and sequencing confirmed that the isolate harboured Klebsiella pneumoniae carbapenemase (KPC)-2, DHA-1 and TEM-1 β-lactamase genes. Both the KPC-2 and DHA-1 genes were transferred to Escherichia coli C600 by transconjugation, and Southern blotting confirmed that these two genes were located on the same plasmid, which was of approximately 56 kb in size. The Enterobacter aerogenes isolate was resistant to carbapenems and other tested antimicrobial agents. The Escherichia coli transconjugant showed reduced susceptibility but not resistance to carbapenems and other β-lactams, indicating the presence of another, possibly permeability-related, resistance mechanism in the clinical isolate.

INTRODUCTION

Klebsiella pneumoniae carbapenemases (KPCs), the most frequent class A carbapenemases, were originally identified from K. pneumoniae isolated from North Carolina. Soon afterwards, these plasmid-mediated carbapenemases were identified in multiple genera and species of the Enterobacteriaceae and non-fermenters. The geographical distribution of blaKPC-producing isolates has spread not only within the USA (Bradford et al., 2004; Bratu et al., 2005), but also in Israel (Navon-Venezia et al., 2006), France (Naas et al., 2005), Greece (Cuzon et al., 2008), China (Shi et al., 2012), Argentina and Brazil (Monteiro et al., 2009).

Enterobacter species are important opportunistic pathogens that can cause nosocomial outbreaks and invasive infections such as bloodstream infections. The treatment of serious infections caused by Enterobacteriaceae producing AmpC cephalosporinases or extended-spectrum β-lactamases (ESBLs) depends mainly on carbapenems. The emergence of carbapenem-resistant strains may have serious consequences because of the limitation of therapeutic choices for patients with invasive Enterobacter aerogenes infections. In the present study, we describe an Enterobacter aerogenes isolate producing KPC-2 carbapenemase and DHA-1 AmpC enzyme, the isolate having been recovered from a patient undergoing hospital treatment.

METHODS

Bacterial strain. The carbapenem-resistant Enterobacter aerogenes WX04 strain was isolated from the sputum of a 69-year-old patient hospitalized in the intensive care unit on 3 July 2010. The patient suffered from primary biliary cirrhosis and had been admitted to the hospital on 4 May 2010. During hospitalization, imipenem was repeatedly administered (total dosage of imipenem received was 70 g). Species level identification of the isolate was confirmed by VITEK cards (bioMérieux) and standardized biochemical tests.

Antimicrobial susceptibility testing. Antibiotic sensitivity testing of Enterobacter aerogenes WX04 was performed by the agar dilution method (Mueller–Hinton agar; Oxoid). The results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009). Interpretations of susceptibility for all antimicrobials tested were based on the CLSI criteria (CLSI, 2009). Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were routinely included in the testing for quality assurance.

PCR amplification and DNA sequence analysis of bla genes. DNA from the clinical isolate and Escherichia coli transconjugant was used as a template in PCR amplification. The primers used to amplify the carbapenemase genes blaKPC, blaIMI, blaVIM, blaSPM, blaOXA-48, blaNDM, blaGES-1, the ESBL genes blaTEM, blaSHV and blaCTX-M, and the plasmid-borne AmpC genes, including those encoding MOX-1, MOX-2, CMY-1–CMY-11, LAT-1–LAT-4, BLE-1, DHA-1, DHA-2, AAC, MIR-1, ACT-1 and FOX–FOX-5b, were as described previously, as were the amplification conditions (Kuai et al., 2010; Smith Moland et al., 2003; Nüesch-Inderbinen et al., 1997; Rasheed et al., 1997; Edelstein et al., 2003; Pérez-Pérez & Hanson, 2002). PCR amplification products were purified and directly sequenced using an ABI 3730 Sequencer (Applied Biosystems) and the sequences were compared with the reported sequences from GenBank using the Basic Local Alignment Search Tool (BLAST) program.
Conjugation experiments, plasmid purification and Southern blotting analysis. Rifampicin-resistant *Escherichia coli* EC600 (LacZ− NaI− RifR) was used as the recipient strain. A conjugation experiment was carried out in mixed-broth cultures as described previously (Shi et al., 2012). *Escherichia coli* transconjugants were selected on Mueller–Hinton agar plates containing 256 µg rifampicin ml−1 (Sigma) plus 1 µg imipenem ml−1. Plasmid DNA from the *Enterobacter aerogenes* strain and its transconjugant was isolated by use of a plasmid DNA midi kit (Qiagen) according to the manufacturer’s instructions. Plasmid DNA extracted from the original isolate and its transconjugant was electroporated on 0.8% agarose gels in the presence of 1.0 Tris-acetate-EDTA buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.3). The DNA was then transferred to a Hybond-N+ membrane (Amersham Bioscience) and cross-linked by use of UV light. Primers of blabKPC-2 and blabDHAA-1 labelled with α-32P were used as specific probes. Meanwhile, the same plasmid DNA was used as a template to confirm the existence of blabKPC-2 and blabDHAA-1 using specific primers.

**Isoelectric focusing of β-lactamases.** Crude cell lysates were prepared by ultrasonication from *Enterobacter aerogenes* WX04 and its transconjugant, as described previously (Navon-Venezia et al., 2006). β-lactamases were characterized by analytical isoelectric focusing (IEF) with the PhastSystem (Pharmacia) according to established methods (Poirel et al., 2004). Cell extracts were analysed by using commercially prepared polyacrylamide gel plates at pH 3.5–9.5 (Amersham-Pharmacia). β-lactamases were visualized by staining the IEF gel with a 0.05% (0.96 mM) solution of nitrocefin (BD Biosciences). The isoelectric points of DHA-1 (7.8), KPC-2 (6.7) and TEM-1 (5.4) were estimated by comparison with those of TEM-1 (5.4), KPC-2 (6.7), SHV-18 (7.6) and ACT-1 (9.0) extracted from ATCC K. pneumoniae 700603, *Enterobacter cloacae* 029M and a clinical isolate of *K. pneumoniae* producing KPC-2 enzyme.

**Analysis of the genetic environment of the blabKPC gene.** The plasmid DNA extracted from the original isolate and the transconjugant was used as a template for PCR. Determination of the genetic environment around the KPC gene was accomplished by using primers CETnF1 (5’-CAT GCC GTA GGT TGT TG TGC CGC-3’) and CETnR1 (5’-GCG GCA GAA GCC AAA ATC G-3’) designed by Gootz et al. (2009). The primer KPC-F1 (5’-CGG CTA ACA AGG ATG ACA AG-3’), which is identical to bp 799–818 of the KPC-2 gene (GenBank accession number AY034847) and the primer KPC-R1 (5’-ACT TAC AGT TGC GGC CTG AGC-3’), which is complementary to bp 179–160 of the KPC-2 gene (GenBank accession number AY034847) as well as sequences, were analysed by the use of the BLAST program.

**RESULTS AND DISCUSSION**

The results of the susceptibility testing are shown in Table 1. The original isolate exhibited resistance to aztreonam as well as to carbapenem, cephalosporin and aminoglycoside antibiotics, but was not resistant to levofloxacin, suggesting a choice for clinical therapy. The MICs of expanded-spectrum cephalosporins and carbapenem were slightly modified after the addition of clavulanic acid. Attempts to transfer reduced imipenem susceptibility from *Enterobacter aerogenes* WX04 to rifampicin-resistant *Escherichia coli* EC600 (LacZ− NaI− RifR) by a mixed-broth mating procedure (Wei et al., 2005) succeeded, indicating that the plasmid carrying the carbapenem resistance determinant possessed its own transfer-associated gene clusters. Mueller–Hinton agar containing 1 µg imipenem ml−1 and 256 µg rifampicin ml−1 was used to select the transconjugants. Transfer of the plasmid to a susceptible *E. coli* strain caused a significant increase in MICs but did not confer the same resistance level of imipenem and meropenem, suggesting that an additional mechanism is involved in carbapenem resistance in the *Enterobacter aerogenes* strain, such as alterations in outer membrane permeability. Plasmid DNA present in *Enterobacter aerogenes* WX04 and its transconjugant were extracted using the Qiagen plasmid midi kit; they all harboured a single plasmid with a size of approximately 56 kb (data not shown). The respective DNA extracts were subjected to specific PCR assays and sequencing for the blabKPC-2 and blabDHAA-1 genes. It was confirmed that the two genes were located on the same 56 kb plasmid; the primers and PCR conditions used were as previously described (Kuai et al., 2010; Smith Moland et al., 2003). Moreover, Southern hybridization revealed a positive signal for the 56 kb plasmid with either the blabKPC-2 or blabDHAA-1 probes (data not shown), and the results of molecular hybridization assays were consistent with those obtained by PCR.

The results of isoelectric focusing showed that *Enterobacter aerogenes* WX04 had three β-lactamases with isoelectric point (pI) values of 5.4, 6.7 and 7.8, while its transconjugant had two β-lactamases with pI values of 6.7 and 7.8. PCR experiments using genomic DNA from the *Enterobacter aerogenes* isolate as a template, followed by sequencing, identified three β-lactamase genes coding for the carbapenemase KPC-2 (pI 6.7), the narrow-spectrum TEM-1 (pI 5.4) and the DHA-1-type AmpC enzyme (pI 7.8). Comparison with the plasmid analysis and transconjugant data showed that the TEM enzyme was not encoded on a plasmid (Fig. 1).

Previous studies of the genetic environment of the blabKPC gene have identified a transposon-associated element, Tn4401, which was considered to be the reason for blabKPC-like gene acquisition and dissemination (Naas et al., 2005). However, in our present work, the genetic environment around blabKPC showed no significant signal was acquired when the plasmid was sequenced with primers CETnF1 and CFTnR1, and that the genetic environment around blabKPC in the original isolate and its transconjugant was not the transposon Tn4401 (Gootz et al., 2009). The assembly results revealed a 2.6 kb sequence that was part of the genetic context of the blabKPC gene, from 5835 bp to 8434 bp in the plasmid pKP048 (GenBank accession no. FJ 28167.1) reported by Shen et al. (2009). Outside this region, sequencing revealed a context for blabKPC with the gene order Tn3-transposase, Tn3-resolvase, ISKpn8, the blabKPC-2 gene, and the ISKpn6-like element from Morganella morganii (Shi et al., 2012) (Fig. 2). These results suggested that blabKPC-2 carried in the *Enterobacter aerogenes* isolate was possibly transferred between different species by the same mobile genetic element. This mode of transmission may increase the chance of bacteria acquiring carbapenem and cephalosporin resistance, causing serious nosocomial infection.
In conclusion, this report identified a carbapenem-resistant *Enterobacter aerogenes* producing DHA-1 AmpC enzyme and KPC-2 carbapenemase that probably resulted from an inter-species transfer of the KPC producer from Hangzhou, Zhejiang (Shen *et al.*, 2009). Its resistance to carbapenem was mainly due to producing KPC-2 carbapenem-hydrolysing β-lactamase, and DHA-1-type AmpC enzyme might contribute to higher levels of carbapenem resistance in accordance with previous studies (Smith Moland *et al.*, 2003; Hossain *et al.*, 2004). Furthermore, KPC-2 and DHA-1 were located on a transferable plasmid, which has the same plasmid profile as seen in *Klebsiella pneumoniae* producing KPC-2 that is prevalent in our hospital (Kuai *et al.*, 2010). This indicated that it has a high potential to spread among clinical isolates and that detection of carbapenem-hydrolysing β-lactamases is urgently needed because carbapenems are one of the few remaining

### Table 1. Antimicrobial susceptibility of *Enterobacter aerogenes* WX04 and its transconjugant in this study

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th><em>Enterobacter aerogenes</em> WX04</th>
<th>Transconjugant <em>Escherichia coli</em> WX04</th>
<th><em>Escherichia coli</em> 600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoperazon</td>
<td>&gt;256</td>
<td>128</td>
<td>0.03</td>
</tr>
<tr>
<td>Cefoperazon/SUL*</td>
<td>&gt;256</td>
<td>64</td>
<td>0.03</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;256</td>
<td>128</td>
<td>0.25</td>
</tr>
<tr>
<td>Piperacillin/TZB†</td>
<td>&gt;256</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>256</td>
<td>32</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefotaxime/CLA‡</td>
<td>128</td>
<td>16</td>
<td>0.06</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>128</td>
<td>32</td>
<td>0.06</td>
</tr>
<tr>
<td>Ceftazidime/CLA‡</td>
<td>32</td>
<td>8</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>256</td>
<td>128</td>
<td>0.50</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>64</td>
<td>16</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Imipenem</td>
<td>32</td>
<td>8</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Imipenem/CLA‡</td>
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<td>2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Meropenem</td>
<td>32</td>
<td>4</td>
<td>&lt;0.03</td>
</tr>
<tr>
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<td>1</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Amikacin</td>
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<td>64</td>
<td>1.00</td>
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<tr>
<td>Levofloxacin</td>
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<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>8</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

*SUL, sulbactam 4 µg ml⁻¹; †TZB, tazobactam 4 µg ml⁻¹; ‡CLA, clavulanic acid 4 µg ml⁻¹.*

In Fig. 1, isoelectric focusing of β-lactamase preparations from *Enterobacter aerogenes* WX04 and its *Escherichia coli* transconjugant. The gel was stained with nitrocefin. Lanes: 1, *Escherichia coli* transconjugant of *Enterobacter aerogenes* WX04 KPC-2 (pI 6.7) and DHA-1 (pI 7.8); 2, *Enterobacter aerogenes* WX04 producing TEM-1 (pI 5.4), KPC-2 (pI 6.7) and DHA-1 (pI 7.8); 3 and 4, pIs of the β-lactamases were calculated by using the known pIs of TEM-1 (pI 5.4), KPC-2 (pI 6.7), SHV-18 (pI 7.6) and ACT-1 (pI 9.0).

In Fig. 2, schematic representation of the novel genetic structure associated with the *bla*KPC-2 gene in *Enterobacter aerogenes* WX04 in China. Open reading frames are shown and their directions of transcription are represented as broad arrows. Tn3-tnpA, Tn3-transposase; Tn3-tnpR, Tn3-resolvase; ISKpn6-like, ISKpn6-like element from *Morganella morganii*.
therapies for infections caused by high-level AmpC- or ESBL-producing Enterobacter species. In order to avoid an epidemic of strains producing KPCs, strict resistance surveillance and drug administration should be emphasized in this area.

REFERENCES


