Detection of clonal KPC-2-producing \textit{Klebsiella pneumoniae} ST258 in Korea during nationwide surveillance in 2011

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This study analysed the characteristics and genetic similarity of recent \textit{Klebsiella pneumoniae} carbapenemase (KPC-2)-producing \textit{Klebsiella pneumoniae} isolates from Korea. Recent laboratory surveillance detected an increase in carbapenemase-producing \textit{Enterobacteriaceae} in Korea. A total of 6 KPC-2-producing \textit{K. pneumoniae} were identified from 277 \textit{Enterobacteriaceae} clinical isolates. All were sequence type (ST) 258 and they had the same pulsotype. They had high MICs for carbapenems and multi-drug resistance. TEM-1, SHV-11 and OXA type \(\beta\)-lactamases were detected in all isolates, whereas CTX-M type \(\beta\)-lactamases and plasmid-mediated AmpC \(\beta\)-lactamase (PABL) were not present. A conjugation experiment failed, but \(\text{bla}_{\text{KPC-2}}\)-harbouring plasmids from the six isolates were used to transform \textit{Escherichia coli} DH5\(\alpha\) by electroporation. Each of the transformants harboured a \(\text{bla}_{\text{KPC-2}}\)-positive approximately 95 kb plasmid, which was typed in the IncFII incompatibility group and co-harboured TEM-1 and OXA-9 \(\beta\)-lactamases. They shared the same restriction profile. This study confirms the emergence of clonal ST258 KPC-2-producing \textit{K. pneumoniae} in some regions of Korea.

INTRODUCTION

The prevalence of carbapenemase-producing \textit{Enterobacteriaceae} has increased recently. These organisms have become a major concern in healthcare institutions because they are often co-resistant to various antibiotics, which leaves few treatment options (Hirsch & Tam, 2010; Kumarasamy et al., 2010). Unlike non-fermenting bacteria that also produce KPC, \textit{Enterobacteriaceae} such as \textit{Klebsiella pneumoniae} constitute the normal human intestinal flora, which acts as a reservoir for these potentially pathogenic bacteria. Carriage may persist for years, so these organisms may spread throughout the healthy population within a community (Schwaber & Carmeli, 2008). Extended-spectrum \(\beta\)-lactamase (ESBL)- and KPC-producing \textit{K. pneumoniae} have become a threat to vulnerable hospitalized patients and potentially to individuals in the wider community (Rodrı́guez-Ban˜o et al., 2008; Schwaber & Carmeli, 2008; Won et al., 2011).

KPC-producing \textit{K. pneumoniae} have spread rapidly worldwide since they were first isolated in New York. Recently, the clone sequence type (ST) 258 was shown to have spread extensively in many countries including the USA, Israel, Greece, France, Colombia, China and the UK (Bradford et al., 2004; Cuzon et al., 2010; Grundmann et al., 2010). KPC-producing \textit{K. pneumoniae} is very difficult to control and often requires a centrally coordinated intervention to combat outbreaks if it is not controlled soon after its emergence (Schwaber et al., 2011).

Prior to 2008, only a few cases of VIM-2-producing \textit{Enterobacteriaceae} had been reported from Korea (Yum et al., 2002; Jeong et al., 2003; Kim & Shin, 2005). However, two sporadic cases of KPC-2-producing \textit{K. pneumoniae} were reported recently. One case (ST11) was isolated in 2009 (Rhee et al., 2010) and the other case (ST258) in 2010 (Roh et al., 2011) from individuals with no history of travel or medical treatment in endemic countries.

In the present study, KPC-2-producing \textit{K. pneumoniae} were isolated during the surveillance of suspected KPC-containing \textit{Enterobacteriaceae}. The aim of this study was to characterize these KPC-2-harbouring \textit{K. pneumoniae} isolates from Korea.

METHODS

Bacterial isolates. Carbapenem-resistant \textit{Enterobacteriaceae} isolates were collected during a national surveillance between November 2010
and October 2011. A total of 277 isolates that were resistant to imipenem or meropenem were referred to our laboratory from 49 hospitals and 2 commercial laboratories. The identifications were confirmed by 16S rRNA sequence analysis. The MICs of imipenem, meropenem and tigecycline were determined using Etest (bioMérieux) according to the manufacturer’s instructions. The modified Hodge test was performed using discs containing 10 µg meropenem. For KPC-producing isolates, the MICs of antimicrobial agents were determined using the agar dilution method. The susceptibility test results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). Colistin and tigecycline susceptibility were determined based on the European Committee on Antimicrobial Susceptibility Testing breakpoints.

PCR amplification and sequencing. PCR and direct sequencing were performed for the blaKPC-2 gene using the primers KPC-F 5’-ATGTCACTGTATCGCCGTCT-3’ and KPC-R 5’-TTTTCAGACCTTTACTGGCCTC-3’ (Bradford et al., 2004). KPC-producing isolates were screened by PCR using primers for blaCTX-M, blasIV, blaeper, blaci, blact, blacta, blactd, armA group genes, blaox-1 group genes, the plasmid-mediated AmpC β-lactamase (PABL)-encoding genes and 16S methylase genes (armA, rmtA, rmtB, rmtC, rmtD) (Doi & Arakawa, 2007; Yoo et al., 2010). All positive PCR products were sequenced and compared with reported gene sequences.

Genetic structures surrounding the blaKPC-2 gene. A series of primers (Cazou et al., 2010) were used for PCR amplification and sequencing to determine the sequences surrounding KPC-2. PCR experiments using Maxime PCR PreMix i-taq (Intron) were performed, which comprised 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 20 s and extension at 72 °C for 30 s, with an additional 7 min extension at 72 °C. The amplified PCR products were sequenced.

Plasmid analysis. PFGE using S1 nuclease (Promega) to digest genomic DNA was performed as described elsewhere, with some modifications (Barton et al., 1995). Gel plugs were digested with 0.1 U S1 nuclease for 30 min at 37 °C. The undigested total DNA of the isolates was separated using a 0.8% agarose gel by PFGE, transferred onto a Hybond-N+ membrane (Amersham Pharmacia Biotech) and hybridized separately with a probe for blaKPC-2. The CDP-Star direct nucleic acid labelling and detection system (GE Healthcare) was used according to the manufacturer’s instructions.

Plasmid DNA was extracted using a Qiagen plasmid midi kit and used to transform Escherichia coli DH5-α. Transformants were selected using MacConkey agar containing ceftazidime (5 mg L−1) and confirmed by blaKPC-2 gene PCR. The plasmid DNA was digested using BanHI and detected by gel electrophoresis on 0.7% agarose at 100 V for 2 h. PCR-based replica typing of the plasmid incompatibility groups was performed as described by Carattoli et al. (2005). A conjugation experiment was attempted using a broth mating-out assay with sodium azide-resistant E. coli J53 as the recipient. Transconjugants were selected with 5 mg ceftazidime L−1 and 150 mg sodium azide L−1.

PFGE and multilocus sequence typing (MLST). The total bacterial DNA was digested using XbaI (New England Biolabs) and the DNA fragments were separated using a CHEF Mapper system (Bio-Rad Laboratories). The conditions were as follows: 14 °C, 6 V cm−1 with pulses of 2.2–63.8 s and a runtime of 21 h. MLST analyses of seven housekeeping genes (rpoB, gapA, mdh, pgi, phoE, infB and tonB) were performed using primers listed in the MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). The STs of the isolates were determined based on the allele sequences in the database.

RESULTS

KPC-2-producing isolates

During the surveillance period, 6 KPC-2-carrying K. pneumoniae isolates (2.2%) were identified from 277 Enterobacteriaceae clinical isolates. All isolates were obtained from urine specimens. Three isolates were found in a tertiary care hospital (hospital A) in Gyeonggi-do, one isolate was found in a tertiary care hospital (hospital B) in Seoul and the remainder were found in a geriatric hospital (hospital C) in Seoul. Four patients were confirmed as having no history of overseas travel or medication, whereas information was not available for the other two patients (the sources of isolates CRKP-26 and CRKP-53). The patients from hospital A and hospital C had no direct contact. The second patient from hospital A was treated in the emergency room and discharged the same day, while the third patient was admitted several months after the discharge of the first patient. There was also no overlap in the hospital-stay period of the two patients reported from hospital C.

Resistance phenotypes and genotypes of KPC-2-producing K. pneumoniae

All of the isolates had high MICs for carbapenems (>32 mg L−1) and multi-drug resistance to amikacin, ciprofloxacin, aztreonam and ceftazidime, whereas they were susceptible to tigecycline and colistin (Table 1). Five of the isolates were also susceptible to gentamicin, whereas CRKP-26 had a high MIC (>256 mg L−1).

All isolates tested strongly positive in the modified Hodge test and were PCR positive for blaKPC-2 and they were confirmed as KPC-2 by sequencing. TEM-1, SHV-11 and OXA-9 β-lactamases were detected in all isolates, whereas CTX-M type β-lactamase and PABL were not found. Only CRKP-26, which was highly resistant to gentamicin, possessed the 16S rRNA methylase, ArmA (Table 1).

Analysis and transferability of KPC-2-carrying plasmids

CRKP-18, CRKP-26 and CRKP-88 harboured plasmids that measured approximately 40 kb, approximately 95 kb and approximately 195 kb in length, respectively, whereas CRKP-51, CRKP-53 and CRKP-62 harboured approximately 40 kb, approximately 95 kb and approximately 145 kb plasmids, respectively. The location of the blaKPC-2 gene was identified by Southern blot hybridization of the S1 nuclease-digested total DNA using a blaKPC-2 probe. KPC-2 was detected in the approximately 95 kb plasmids of all isolates (Fig. 1a). A conjugation experiment was attempted via a broth mating-out assay using sodium azide-resistant E. coli J53 as a recipient, but none of the plasmids were transferable to E. coli. However, blaKPC-2 harbouring plasmids from all isolates were transferred to E. coli DH5-α via electro-transformation. Plasmid characterization was performed using the transformants, including

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replicon typing, restriction pattern analysis and gene profile analysis. PCR-based replicon typing of the major plasmid incompatibility groups showed that 
\( \text{bla} \text{KPC-2} \)-carrying plasmids belonged to one incompatibility group, IncFII. Restriction profile analyses of the approximately 95 kb plasmids were performed using the restriction enzyme BamHI, which detected only one type of restriction pattern (Fig. 1b).

The 
\( \text{bla} \text{KPC-2} \) genes of all isolates were located on Tn4401a, which was 100 bp shorter than the Tn4401b fragment between ISKpn7 and 
\( \text{bla} \text{KPC-2} \). PCR products of the expected size were obtained using primer pairs specifically for the different genes found on Tn4401, which suggested a similar genetic organization in all of the isolates tested.

**Molecular epidemiology of KPC-2-producing *K. pneumoniae***

MLST detected only one ST according to the *K. pneumoniae* MLST database and all isolates were identified as ST258 (allelic profile 3-3-1-1-1-1-79). Molecular typing by PFGE identified one pulsotype with high similarity (90 %; Fig. 2). Three isolates (CRKP-51, CRKP-53 and CRKP-62) from hospitals B and C were identical, while the three remaining isolates (CRKP-18, CRKP-26 and CRKP-88) from hospital A shared 100 % identity.

**DISCUSSION**

The emergence of KPC-2-producing *Enterobacteriaceae* is a major clinical and public-health concern worldwide. Clonally related *K. pneumoniae* ST258 has caused outbreaks in the USA and Israel (Kitchel *et al.*, 2009; Schwaber *et al.*, 2011), and has spread throughout the world (Cuzon *et al.*, 2010).

In Korea, the prevalence of carbapenem-resistant *Enterobacteriaceae* is <1 %, while resistance to imipenem in *E. coli* and *K. pneumoniae* isolates was reported as 0.1 and 0.5 %, respectively, in 2008 (Lee *et al.*, 2011). Until recently, KPC-producing *Enterobacteriaceae* were reported very rarely in Korea. However, after laboratory surveillance was established to monitor carbapenemase-producing *Enterobacteriaceae* in accordance with the revised Infectious Disease Prevention Acts (December 2010), various carbapenemase-producing *Enterobacteriaceae*, including KPC-2, NDM-1, IMP-1 and VIM-2, were detected (KCDC, 2011). Moreover, six genetically related KPC-2-producing isolates were isolated within 1 year.

The origins of these isolates could not be determined due to insufficient patient history and epidemiological data. Based on the patient information, these cases were determined to be epidemiologically unrelated, although all shared high genetic similarity (90 %). Transmission within a hospital might occur from KPC-2 carrier patients in the hospital, healthcare workers or medical devices contaminated with KPC-2-producing *K. pneumoniae*.

**Table 1. Characteristics of KPC-2-producing *K. pneumoniae* isolates**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Date of isolation</th>
<th>Hospital*</th>
<th>Specimen</th>
<th>-Lactamase</th>
<th>SHV</th>
<th>TEM</th>
<th>OXA</th>
<th>CTX-M</th>
<th>PAB</th>
<th>IPM</th>
<th>AMR</th>
<th>GEN</th>
<th>IMP</th>
<th>MEM</th>
<th>ATM</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRKP-18</td>
<td>Dec 2010</td>
<td>A</td>
<td>Urine</td>
<td>KPC-2</td>
<td>1</td>
<td>11</td>
<td>9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>64</td>
<td>32</td>
<td>256</td>
<td>128</td>
<td>64</td>
<td>&gt;256</td>
</tr>
<tr>
<td>CRKP-51</td>
<td>April 2011</td>
<td>B</td>
<td>Urine</td>
<td>KPC-2</td>
<td>1</td>
<td>11</td>
<td>9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>64</td>
<td>32</td>
<td>256</td>
<td>128</td>
<td>&gt;256</td>
<td></td>
</tr>
<tr>
<td>CRKP-53</td>
<td>April 2011</td>
<td>C</td>
<td>Urine</td>
<td>KPC-2</td>
<td>1</td>
<td>11</td>
<td>9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>64</td>
<td>32</td>
<td>256</td>
<td>128</td>
<td>&gt;256</td>
<td></td>
</tr>
<tr>
<td>CRKP-62</td>
<td>April 2011</td>
<td>C</td>
<td>Urine</td>
<td>KPC-2</td>
<td>1</td>
<td>11</td>
<td>9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>64</td>
<td>32</td>
<td>256</td>
<td>128</td>
<td>&gt;256</td>
<td></td>
</tr>
<tr>
<td>CRKP-88</td>
<td>Sept 2011</td>
<td>A</td>
<td>Urine</td>
<td>KPC-2</td>
<td>1</td>
<td>11</td>
<td>9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>64</td>
<td>32</td>
<td>256</td>
<td>128</td>
<td>&gt;256</td>
<td></td>
</tr>
</tbody>
</table>

*Hospitals A and B were tertiary care hospitals, hospital C was a geriatric hospital.

- AMK, Amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CL, colistin; CTX, ceftriaxone; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TGC, tigecycline.
The transfer of plasmids is the crucial mechanism that underlies the increase in carbapenem-resistant Enterobacteriaceae. Plasmids isolated from KPC-producing Enterobacteriaceae belong to various incompatibility groups (IncN, IncA/C, IncHI2, IncFII and ColE-like) (Carattoli, 2009; Cuzon et al., 2010; Gomez et al., 2011). All of the KPC-2-carrying plasmids in the present study were IncFII plasmids, which demonstrated their high frequency among the typed resistance plasmids. The host ranges of these plasmids are limited to Enterobacteriaceae genera, whereas IncN, IncP and IncA/C plasmids have a wider range of hosts (Carattoli, 2009). IncFII group plasmids that carry KPC-2 have been reported in Israel. A pKpQIL plasmid, which was identified in an isolate from a KPC-3-carrying K. pneumoniae ST258 outbreak, was self-transmissible and it conferred high carbapenem MICs (Leavitt et al., 2010). The KPC-2-producing isolates in the present study also had high MICs for carbapenems, similar antibiograms (susceptible to gentamicin and colistin), similar plasmid sizes (95 kb) and restriction profiles (Goren et al., 2010), but our isolates were not self-transmissible.

Two of the isolates identified in this study were derived from one geriatric hospital. Geriatric hospitals appear to be contributory factors to outbreaks of KPC- and ESBL-producing Enterobacteriaceae (Won et al., 2011). In Korea, geriatric hospitals have higher rates of ESBL in K. pneumoniae and E. coli compared with tertiary care hospitals (Yoo et al., 2010). Thus, guidance for the control of KPC-producing Enterobacteriaceae should be expanded to include geriatric hospitals.

Overall, the present study suggests that KPC-2-producing K. pneumoniae ST258 was introduced into Korea by an unknown route and has begun to spread throughout the country. Coordinated regional efforts involving tertiary hospitals, geriatric hospitals (or long-term care facilities) and public-health departments are crucial for preventing a nationwide outbreak (Won et al., 2011). Furthermore, continuous surveillance of KPC-producing Enterobacteriaceae and the

Fig. 1. Analysis of KPC-2-carrying plasmids from six K. pneumoniae isolates. (a) PFGE after digestion of DNA with S1 nuclease (left panel) and Southern blot with KPC-2 probe (right panel). Lanes: M, Lambda ladder gel marker; 1, CRKP18; 2, CRKP26; 3, CRKP51; 4, CRKP53; 5, CRKP62; 6, CRKP88. (b) BamHI restriction of KPC-2-carrying plasmids from E. coli transformants (left panel) and Southern blot with KPC-2 probe (right panel). Lanes: M, λ HindIII marker; 1, CRKP18; 2, CRKP26; 3, CRKP51; 4, CRKP53; 5, CRKP62; 6, CRKP88.

Fig. 2. Dendrogram analysis of XbaI-digested DNA from KPC-2-carrying K. pneumoniae isolated in South Korea.
education of laboratory workers, clinicians and infection control personnel are necessary.

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