Molecular Characterization of Newly Emerged \textit{bla}_{KPC-2}-Producing \textit{Klebsiella pneumoniae} in Singapore

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In Asia, \textit{bla}_{KPC} detection has been limited to East Asia and not yet seen in Southeast Asia. We report four \textit{bla}_{KPC-2}-containing \textit{Klebsiella pneumoniae} isolates from two different hospitals in Singapore. All isolates belonged to strain type 11 (ST11) and were indistinguishable by pulsed-field gel electrophoresis (PFGE). \textit{bla}_{KPC-2} was located on nonconjugative plasmids and flanked by mobile genetic structures composed of a partial Tn4401 transposon and a Tn3-based transposon which previously have been described only in Chinese isolates.

In Asia, \textit{Klebsiella pneumoniae} carbapenemase (KPC) detection has been limited to East Asia (2, 4, 12, 13) but as yet has not been reported in Southeast Asia. Carbapenem resistance occurs in less than 1% of hospital-associated \textit{Klebsiella} infections in Singapore except for resistance to ertapenem, which occurs in approximately 3% of cases. This is usually mediated by expression of extended-spectrum $\beta$-lactamases (ESBL) or AmpC cephalosporinases combined with porin mutations (7). Carbapenemases, including New Delhi metallo-$\beta$-lactamase (NDM-1), have been detected with increasing frequency over recent years, but detection remains sporadic. In June and July 2011, four \textit{bla}_{KPC-2}-containing \textit{K. pneumoniae} isolates were identified in epidemiologically unrelated patients from two different hospitals in Singapore.

Species level identification was performed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics, Germany). Antimicrobial susceptibility testing was performed with a Vitek-2 instrument, and carbapenem MICs were confirmed with the Etest (Biomerièux, Marcy l’Etoile, France). Susceptibility was defined according to the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Carbapenemase production was detected using KPC and metallo-$\beta$-lactamase (MBL) confirmation discs (Rosco Diagnostica A/S, Taastrup, Denmark).

The presence of \textit{bla}_{KPC} was detected by PCR using \textit{bla}_{KPC}-specific primers (forward primer, 5’-CGTTGACGGCCAATCC-3’; reverse primer, 5’-ACCGCTGGCAAGCTGG-3’) with confirmation by full-length \textit{bla}_{KPC} sequencing. The isolates also underwent PCR screening for other transmissible \textit{bla} genes, including those encoding serine carbapenemases (GES-1 to -5 and -7), MBLs (VIM type, IMP type, and NDM-1), oxacillinases (OXA-1, -2, -7, -8, and -48), ESBLs (TEM, SHV, and CTX-M types), and plasmid-located AmpC cephalosporinases (CMY type and DHA-1), and for 16S rRNA methylase genes (\textit{rmtA}, \textit{rmtB}, \textit{rmtC}, \textit{rmtD}, and \textit{npmA}) which cause high-level aminoglycoside resistance.

Clonal relatedness of the isolates was investigated using multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). MLST was performed using the protocol developed by the Institut Pasteur (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). DNA for PFGE was prepared as previously described (6) using SpeI (New England BioLabs, Ipswich, MA). Digested DNA was separated on a 1.2% agarose gel (Bio-Rad, La Jolla, CA) and 0.5% Tris-borate-EDTA buffer by using a CHEF-DRII system (Bio-Rad) at 14°C and 6 V/cm with alternating pulses at a 120° angle in a 10- to 40-s pulse time gradient for 22 h.

Plasmids were isolated using the QIAprep spin miniprep kit (Qiagen GmbH, Hilden, Germany). Estimation of plasmid sizes was performed by SI nuclease digestion followed by gel electrophoresis (1). Southern hybridization analysis for \textit{bla}_{KPC} was carried out using the digoxigenin (DIG) DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany). Conjugation experiments were performed between \textit{K. pneumoniae} isolates and azide-resistant recipient \textit{Escherichia coli} J53. Plasmid DNA extracted from the KPC producers was introduced by electroporation into \textit{E. coli} DH5a using Gene Pulser Xcell (Bio-Rad, Hercules, CA). Transconjugants and transformants were selected on Luria-Bertani (LB) agar containing sodium azide (50 mg/liter) supplemented with cefazidime (50 mg/liter) or imipenem (1 mg/liter) (14). The immediate genetic environment upstream and downstream of \textit{bla}_{KPC} was determined by using published primers that detected ISKpn7 and ISKpn6 (8) and ISKpn8 and ISKpn6-like insertion sequences (13). Amplified segments were sequenced to confirm gene identity.

Three \textit{K. pneumoniae} isolates were from urine specimens and resulted in clinical infection. The fourth isolate was identified as part of a laboratory stool surveillance screening project performed after \textit{bla}_{NDM-1}-producing \textit{Enterobacteriaceae} were detected at that hospital. None of the patients had traveled overseas within the previous year, and all had prolonged hospital admissions prior to detection of the KPC-producing organisms. There were no epidemiological links between the patients.

All four isolates demonstrated a positive combined disk test with meropenem-boronic acid/meropenem, indicative of serine carbapenemase production. Full gene sequencing of \textit{bla}_{KPC} revealed 100% homology to the KPC-2 gene for all isolates.

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(GenBank accession no. FJ628167.2). The KPC-2 producers displayed a multidrug-resistant phenotype (Table 1) with consistent susceptibility only to colistin and tigecycline. Only one isolate was susceptible to aminoglycosides. All isolates carried ESBL genes and one also carried plasmid-mediated AmpC $\beta$-lactamase bla$_{KPC-2}$. None of the isolates were positive for MBLs or OXA-type $\beta$-lactamases. Only one possessed the armA gene, but three of the four isolates had an amikacin MIC of $>256$ mg/l, suggesting an alternative aminoglycoside resistance determinant, such as the more commonly encountered acetyltransferases, nucleotidyltransferases, and phosphotransferases (5).

The KPC producers were of identical sequence type, ST11, and had indistinguishable PFGE patterns, indicating that the isolates were clonal in nature despite being found at two different hospitals. ST11, a single-locus variant of the international epidemic clone 258, is the dominant clone in Zhejiang, China, with 64% of the KPC-producing isolates being of this sequence type (11).

The isolates carried a variable number (one to three) of plasmids ranging from approximately 10 to 30 kb in size. Southern hybridization reveals that bla$_{KPC-2}$ is located on a plasmid of approximately 20 kb, which is present in all four isolates (data not shown). In our hands, the bla$_{KPC-2}$-bearing plasmids appear to be nonconjugative, since neither transconjugants nor transformants could be observed.

Genetic mapping around the bla$_{KPC-2}$ gene of the isolates revealed that they were genetically organized in the same manner as the bla$_{KPC-2}$-bearing plasmid pK048 (GenBank accession no. FJ628167.2), where the bla$_{KPC-2}$ is flanked upstream by ISKpn8 and downstream by ISKpn6-like insertion sequence elements. Sequencing of ISKpn8 and the ISKpn6-like element of the isolates reveals a 100% nucleotide identity to pK048. This structure is chimerically composed of a Tn4401 partial segment and a Tn3-based transposon. It is considered to be distinctly different from Tn4401 associated with most KPC-2 genes and has been reported only in China (9, 13). The mobile structure has been described in conjugative and nonconjugative plasmids containing either bla$_{KPC-2}$ or bla$_{KPC-3}$ and in three different genera: Klebsiella, E. coli, and Citrobacter (8, 13). This provides insights into the diverse means which bla$_{KPC}$ may use to move between different genetic elements. Emerging data suggest that the propensity for widespread dissemination of bla$_{KPC}$ is complex and involves both the spread of efficient epidemic clones of K. pneumoniae, contributed to by human population dynamics, and the presence of bla$_{KPC}$ on mobile genetic elements. Rearrangements due to novel insertion sequences further affect expression of bla$_{KPC}$ and its ability to transfer between different plasmids and Gram-negative organisms (3). Our findings seem to suggest dissemination from an ancestral strain on mainland China; however, two of the isolates were derived from non-Chinese patients without history of overseas travel, thus local dissemination must be occurring in Singapore. This is a concerning hypothesis supported by the clonality of the isolates.

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**REFERENCES**

10. Reference deleted.

**TABLE 1 Characteristics and susceptibility profiles of KPC-2-producing isolates**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th><em>β</em>-Lactamase</th>
<th>MIC (mg/l)*</th>
<th>CIP</th>
<th>LEV</th>
<th>CHL</th>
<th>TGC</th>
<th>COL</th>
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<tbody>
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<td>HospitalA-1</td>
<td>Stool</td>
<td>TEM-1, SHV-11, CTX-M-15</td>
<td>32, 32, 256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<tr>
<td>HospitalA-2</td>
<td>Urine</td>
<td>TEM-1, SHV-11, CTX-M-3</td>
<td>32, 32, 256</td>
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<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
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*IMP, imipenem; MEM, meropenem; ETP, ertapenem; CTX, cefotaxime; CAZ, ceftazidime; AZT, aztreonam; GEM, gentamicin; AMK, amikacin; CIP, ciprofloxacin; CHL, chloramphenicol; TGC, tigecycline; COL, colistin.*