Emergence of KPC-2 and KPC-3 in carbapenem-resistant *Klebsiella pneumoniae* in an Israeli hospital

**Running title**: KPC in carbapenem-resistant *K. pneumoniae* in Israel

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Abstract

Carbapenem resistance due to KPC has rarely been observed outside the US. We noticed a sharp increase in carbapenem-resistant *Klebsiella pneumoniae* possessing KPC in Tel Aviv Medical Center from 2004 to 2006. 60% of the isolates belonged to a single clone susceptible only to gentamicin and colistin and carried *bla*KPC-3 while almost all other clones carried *bla*KPC-2. This rapid dissemination of KPC outside the US is worrisome.
Carbapenem resistance in *Klebsiella pneumoniae* does not occur naturally and is mainly due to the presence of acquired carbapenem-hydrolyzing β-lactamases (16). KPC-type enzymes in carbapenem-resistant *K. pneumoniae* was first reported in 2001 in North Carolina (23), and until 2005 the geographical distribution of these enzymes in *Enterobacteriaceae* in general and *K. pneumoniae* specifically was limited to the Eastern United States (2, 4, 18, 22), where KPC-producing *K. pneumoniae* become a frequent nosocomial pathogen (3, 9). Outside of the US, KPC-producing *K. pneumoniae*, have been reported in only three patients; the first case was reported in 2005 in France, had a US origin (14), and more recently a case in Colombia and an additional one in China (20, 21).

KPC-2 and KPC-3 carbapenemase have been observed even more rarely among other gram-negative bacteria, including *Enterobacter* spp., *E. coli*, and *Serratia marcescens* (9). Outside of the US, KPC-2 has been observed once in *S. marcescens* isolated from China (25) and in our hospital in *E. coli* strains (15), and during the same year also in an *Enterobacter cloaca* outbreak strain in our neonatal intensive care unit (6). KPC-3 has never been reported outside the US.

All the carbapenem-resistant *K. pneumoniae* isolates identified in the clinical laboratory of our hospital were collected from January 2004 to December 2006. In this study all *K. pneumoniae* isolates manifesting carbapenem resistance were genotyped and analyzed for the presence of *bla*KPC. The results presented suggest the rapid emergence of KPC in *K. pneumoniae* isolates, affecting multiple clones, and leading to the emergence of carbapenem resistance in *K. pneumoniae*.

During the 3-year study period, from January 2004 to December 2006, a total of 4,149 single-patient *K. pneumoniae* isolates were identified in our hospital. Identification of strains and susceptibility testing were performed using the Vitek 2 automated system (bioMerieux, Marcy, France), with AST-GN09 card for the identification of gram-negative bacilli. Fifty-one isolates (1.2%) were carbapenem resistant, defined by resistance to imipenem and/or meropenem. Sites of
isolation included urine (n=19), body fluids (n=10), wounds (n=9), catheter tips (n=6), blood (n=4) and respiratory tract (n=3). For all carbapenem-resistant isolates, resistance to imipenem, meropenem and ertapenem was confirmed using agar dilution, according to the Clinical Laboratory Standards (8). Susceptibility testing for colistin and tigecycline was performed via Etest, according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). The genetic relatedness of all carbapenem-resistant \textit{K. pneumoniae} strains was determined by pulsed-field gel electrophoresis (PFGE) analysis. DNA preparation and SpeI cleavage were performed as described previously (15) and chromosomal restriction fragments were documented and compared.

During 2004 and 2005, carbapenem-resistant \textit{K. pneumoniae} was isolated from a total of six patients, while during 2006 this number increased dramatically to 45 unique-patient isolates. The annual proportions of isolates resistant to carbapenems were 0.4%, 0.07%, and 3.1%, respectively, for the three years of the study. PFGE of all 51 resistant isolates indicated the presence of 12 different genetic clones affecting one-to-three patients each, and a major clone (clone Q) affecting 31 (60%) of the cases (Figure). Thus, 75% of carbapenem-resistant \textit{K. pneumoniae} isolates in our study represent a clonal transmission, while 25% represents different clones. All clones were resistant to all cephalosporins, aztreonam, ertapenem, imipenem and/or meropenem, and to aminoglycosides. Resistance to aminoglycosides varied; eight clones were susceptible only to amikacin and four clones, including the major clone (clone Q), were susceptible only to gentamycin. Two clones were susceptible to ciprofloxacin, but all were susceptible to colistin. Although clone Q was found to be the major clone in the hospital, its isolation did not occur in clusters of space or time (with the exception of 7 cases isolated from one ward); it was isolated from 15 different wards, over a span of 11 months.
In order to identify the molecular mechanism related to carbapenem resistance in *K. pneumoniae* in our hospital, two isolates- isolate 469 (clone P) and isolate 490 (clone Q) representing two different antibiotic susceptibility profiles (Table), were selected for detailed molecular characterization. The presence of imipenem-hydrolyzing activity in cell extracts was demonstrated by streaking the tested strains away from an imipenem disk placed on a lawn inoculum of a susceptible *E. coli* ATCC 25922 as described previously (24). An imipenem-susceptible *K. pneumoniae* clinical strain was used as a negative control for carbapenemase production. Imipenem hydrolyzing activity measured spectrophotometrically at 299 nm showed specific activities of 44 and 46.5 mU/mg (U=µmole imipenem/min) for isolates 469 and 490, respectively. Beta lactamases in cell-free extracts of the two isolates examined by isoelectric focusing (IEF) demonstrated two nitrocefin-positive bands focusing at pI 6.7 and at pI 7.5 for *K. pneumoniae* 469, and three bands focusing at pIs 5.4, 6.7 and 7.6 for *K. pneumoniae* 490.

PCR screening was performed on cell lysates for identification of the carbapenemase genes using specific *bla* primers designed for identifying known β-lactamase genes including *bla*<sub>OXA</sub> (OXA-23, -24, -40, -58) (1, 10, 17), *bla*<sub>KPC</sub>, *bla*<sub>SME</sub>, *bla*<sub>IMI</sub>, *bla*<sub>NMC</sub> (5, 15) *bla*<sub>GES</sub>, *bla*<sub>IMP</sub> (23), *bla*<sub>VIM</sub> (13) *bla*<sub>SIM</sub> (12), *bla*<sub>GIM</sub> and *bla*<sub>SPM</sub> (7). The two *K. pneumoniae* isolates were found to carry *bla*<sub>KPC</sub>. PCR products were cloned and sequenced as described previously (15). The nucleotide acid and deduced protein sequences of both isolates were analyzed and identified as KPC-2 in strain 469 and KPC-3 in strain 490, corresponding to the beta lactamase with the experimental pI of 6.7.

In order to verify whether the carbapenem-resistance phenotype in *K. pneumoniae* is plasmid-encoded, plasmid DNA was purified using NucleoBond PC 100 plasmid Midi kit (Macherey-Nagel, Germany) and *E. Coli* GeneHogs (Invitrogen, U.K) and transformants were selected on LB agar plates with ampicillin (100 µg/ml). Selected transformed colonies were subjected to antibiotic
susceptibility testing (Table), IEF and PCR screening for the identification of carbapenemases that were acquired upon transformation. Transfer of the \textit{bla}_{KPC}-encoding plasmids raised the MICs of extended-spectrum cephalosporins, aztreonam and carbapenems compared to the susceptible \textit{E. coli} GeneHogs recipient strain, but none of the transformants became resistant to imipenem or meropenem (Table). This observation suggests that the background of the strain is important for phenotypic resistance, and that additional mechanisms such as porin alterations that reduce the entry of carbapenems (11), are involved in carbapenem resistance in these strains. IEF confirmed by PCR and sequencing analysis using plasmid DNA from transformants supported co-transmission of \textit{bla}_{OXA-4} and \textit{bla}_{CTX-M-10} with \textit{bla}_{KPC-2} in isolate 469, and co-transmission of \textit{bla}_{TEM-1} and \textit{bla}_{KPC-3} in isolate 490, suggesting that these genes identified in the clinical strains were encoded on the \textit{bla}_{KPC}-carrying plasmid in each.

We screened all 51 carbapenem resistant \textit{K. pneumoniae} isolates for the presence of \textit{bla}_{KPC}. \textit{bla}_{KPC} was not found in any carbapenem resistant \textit{K. pneumoniae} strains in 2004 (Figure). Cell-free extracts of all the non-KPC-producing strains were assayed for their ability to hydrolyze imipenem in a spectrophotometric assay using imipenem as a substrate and gave negative results suggesting that carbapenem resistance in these isolates did not involve a carbapenem-hydrolyzing enzyme. 43 of 46 (93\%) of the isolates from 2005 to 2006 carried \textit{bla}_{KPC}. All isolates belonging to clone Q (31 isolates) possessed \textit{bla}_{KPC-3} while the other isolates belonging to six different pulsotypes possessed mainly \textit{bla}_{KPC-2} (except for two isolates with \textit{bla}_{KPC-3}). KPC-3 and KPC-2 differ in only one amino acid (H272Y) thus; the co-existence of these two enzymes in our hospital is not surprising and may represent one mutational event followed by clonal spread. The emergence of KPC and its rapid spread after introduction to the hospital are worrisome findings, as therapeutic choices against these pan-resistant organisms are limited. It is possible that the prevalence of KPC-producing \textit{K.
*pneumoniae* could have been underestimated in this study due to the fact that only carbapenem resistant isolates (either imipenem or meropenem or both) were included, and KPC-harboring *K. pneumoniae* strains that did not exhibit carbapenem resistance MICs were missed in the Vitek2 as has been shown previously (19). Nevertheless this study has shown for the first time the rapid dissemination of carbapenem-resistant *K. pneumoniae* due to KPC-2 and KPC-3 outside the US. Given reports of the presence of *blakpc* from 3 continents, laboratories, clinicians, infection control personnel and administrators alike should be alerted to design measures for early identification and control of organisms bearing this resistance gene.
REFERENCES


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FIGURE LEGENDS

FIGURE Molecular epidemiology and emergence of KPC in carbapenem-resistant *K. pneumoniae* strains in Tel Aviv Medical Center during 2004-2006. In 2004 four pan-resistant carbapenem-resistant clones susceptible only to amikacin and colistin were identified; in 2005, KPC emerged in carbapenem-resistant *K. pneumoniae*; in 2006, nine clones existed; two appeared previously in 2004-05 and lacked KPC, and seven, including clone Q, the major clone that emerged in February 2006 possessed KPC. Four of the 7 clones (including clone Q) were susceptible only to gentamycin and colistin and three clones were susceptible only to amikacin and colistin.
TABLE 2 Antimicrobial susceptibility patterns of carbapenem-resistant *K. pneumoniae* strains 469 and 490 and their respective transformants (T)

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (µg/ml)</th>
<th></th>
<th></th>
<th></th>
<th>E. coli Gene Hogs</th>
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<tr>
<td></td>
<td><em>K. pneumoniae</em> 469</td>
<td>T-469</td>
<td><em>K. pneumoniae</em> 490</td>
<td>T-490</td>
<td></td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
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<td>&lt;4</td>
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<tr>
<td>Ceftriaxone</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>8</td>
<td>&lt;1</td>
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<tr>
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<td>&gt;64</td>
<td>&gt;64</td>
<td>16</td>
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<tr>
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<td>32</td>
<td>&gt;64</td>
<td>4</td>
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<td>&gt;64</td>
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<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
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</tr>
<tr>
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<td>&gt;128</td>
<td>&gt;128</td>
<td>64</td>
<td>&lt;4</td>
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<tr>
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<td>Zone (mm)</td>
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<td>-------------</td>
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</tr>
<tr>
<td>Imipenem</td>
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<td>KPC-2 OXA-4, CTX-M-10</td>
<td>KPC-3 TEM-1</td>
<td>KPC-3 TEM-1</td>
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