The Molecular Epidemiology of KPC-producing Klebsiella pneumoniae Strains in Israel, Sequence Types and Plasmid Analyses

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Abstract

Sporadic isolates of carbapenem-resistant KPC-2-producing *Klebsiella pneumoniae* were isolated in Tel-Aviv Medical Center during 2005-2006, parallel to the emergence of the KPC-3-producing *K. pneumoniae* sequence type 258 (ST 258). We aimed to study the molecular epidemiology of these isolates and to characterize their *bla*KPC-carrying plasmids and their origin.

Ten isolates were studied (8 KPC-2 and two KPC-3-producing). All isolates were extremely drug resistant. They possessed *bla*KPC gene and varied in their additional beta-lactamase content. The KPC-2-producing strains belonged to three different sequence types: ST 340 (n=2), ST 277 (n=2) and a novel sequence type, ST 376 (n=4). Among KPC-3-producing strains, a single isolate (ST 327) different from ST 258 was identified, but both strains carried the same plasmid (pKpQIL). The KPC-2-encoding plasmids varied in size (45-95 kb) and differed amongst each of the STs.

Two of the *Klebsiella bla*KPC-2-carrying plasmids were identical to plasmids from *Escherichia coli* suggesting a common origin of these plasmids. These data indicates that KPC evolution in *K. pneumoniae* is related to rare events of interspecies spread of *bla*KPC-2-carrying plasmids from *E. coli* followed by limited clonal spread, whereas KPC-3 carriage in this species is related almost strictly to clonal expansion of ST-258 carrying pKpQIL.
Introduction

KPC-producing carbapenem-resistant *Klebsiella pneumoniae* strains have been increasingly reported worldwide. The first KPC-producing *K. pneumoniae* identified in Tel-Aviv Medical Center, isolated in October 2005, was a KPC-2-producing strain. During 2006 an extremely drug resistant (XDR) KPC-3-producing carbapenem-resistant *K. pneumoniae* clone, ST (sequence type) 258 (PFGE type Q) emerged causing a nationwide outbreak (9). Additional sporadic KPC-2-producing clinical isolates of XDR *K. pneumoniae* were identified during 2006 (9), and were recently reported from another hospital in Northern Israel (7).

The epidemiology and clinical impact of the Israeli KPC-producing *K. pneumoniae* was described previously in several studies (12, 16, 17). The major clone of *K. pneumoniae* (previously referred to as PFGE type Q) belongs to ST 258 which initially emerged in the United-States (8). Since then, clinical isolates belonging to this clone have been detected in numerous geographic regions (13), carrying different *bla*<sub>KPC</sub> alleles. In the United States for example, isolates belonging to ST 258 possess both *bla*<sub>KPC-2</sub> and *bla*<sub>KPC-3</sub> (8). Other places report the existence of a single allele carried by this clone such as *bla*<sub>KPC-2</sub> in Norway and Greece (15), Poland (1) and Finland (14) and *bla*<sub>KPC-3</sub> in the United-Kingdom (18), Sweden (15) and Italy (5).

Molecular studies on the *bla*<sub>KPC</sub>-carrying plasmid of *K. pneumoniae* ST-258 in Israel showed that it harbored a 105-kb *bla*<sub>KPC-3</sub>-carrying self-transmissible plasmid, pKpQIL (10) that differed from the plasmids carried by the genetically-related *K. pneumoniae* ST 258 isolates in the United States (12).

The co-existence of KPC-2-carrying *K. pneumoniae* isolates in our hospital along with KPC-3-carrying *K. pneumoniae* ST 258 lead to this study. We aimed to investigate the molecular epidemiology of these strains, examine their evolutionary
relatedness, to compare their $bla_{KPC}$-carrying plasmids, and to facilitate the understanding of the origin of these plasmids in *K. pneumoniae* by comparing them to $bla_{KPC-2}$-carrying plasmids from other *Enterobacteriacae* in our hospital, such as KPC-producing *Escherichia coli*. 
Materials and Methods

Study isolates

Eight carbapenem-resistant KPC-2-producing *K. pneumoniae* clinical isolates that consist the entire collection of KPC-2 isolates isolated in the clinical laboratory of Tel-Aviv Medical Center during 2005-2006 were studied; all of them showed non-Q pulsotypes (9). Two additional KPC-3-producing *K. pneumoniae* isolates were studied; a single non-Q strain and a representative ST 258 (PFGE type Q) KPC-3-producing isolate (Kpn557) (8). Seven KPC-2-producing *E. coli* isolates isolated during the same time period in our hospital (6) were used for plasmid comparison. *E. coli* Genehogs (Invitrogen Corp., Dorset, United Kingdom) was used as a recipient strain in the transformation experiments.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using a Vitek2 automated system (bioMerieux, Marcy l’Etoile, France). Resistance to imipenem, meropenem, and ertapenem was evaluated using agar dilution and interpreted according to the Clinical and Laboratory Standards Institute (4). Susceptibility testing for colistin and tigecycline was performed by Etest according to the manufacturer’s instructions (AB Biodisk, Solna, Sweden). Colistin breakpoint for susceptibility was ≤4 µg/ml according to the British Society of Antimicrobial and Chemotherapy (BSAC) criteria, and MICs for tigecycline were defined based on the United States Food and Drug Administration breakpoint criteria for *Enterobacteriaceae* (susceptible, ≤2 µg/ml; intermediate, 4 µg/ml; resistant, ≥8 µg/ml).
Determination of genetic relatedness

The genetic relatedness of all carbapenem-resistant *K. pneumoniae* strains was determined by pulsed-field gel electrophoresis (PFGE) analysis and by multilocus sequence typing (MLST). PFGE was performed as previously described (9). Chromosomal restriction fragments were documented and compared using GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). MLST was performed and analyzed using the *K. pneumoniae* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

PCR for determination of antibiotic resistance genes

The identification of *bla* genes (including: *bla*TEM, *bla*SHV, *bla*CTX-M and *bla*OXA) and *bla*KPC genes in all isolates was determined by PCR as described previously (10).

Plasmid analysis and transformation

Plasmid DNA was purified using a NucleoBond PC 100 plasmid midi-kit (Macherey-Nagel GmbH, Duren, Germany). Plasmids isolated from clinical isolates of *K. pneumoniae* and *E. coli* strains and their transformants were digested with different restriction endonucleases such as BglII, SmaI and EcoRV (New England Biolabs, Boston, MA) and their restriction pattern was compared. Plasmids were transformed by electroporation into the *E. coli* Genehogs strain. Transformants possessing *bla*KPC were subjected to antibiotic susceptibility testing and further molecular characterization. Plasmids sizes were determined using S1 treatment following PFGE (10), and compared using GelCompar II software.
Results and Discussion

Epidemiology and genetic relatedness of carbapenem-resistant *K. pneumoniae*

Genotyping of all the KPC-2-producing isolates revealed that they were genetically different (<85% identity) from the KPC-3-producing ST 258 (PFGE type Q). These isolates belonged to three clusters: R (2 isolates), P (2 isolates), and S (4 isolates). Isolates 588 and 365 showed 91.7% and 86.3% identity with PFGE types R and S, respectively and were designated R′ and S′ (Figure 1). Isolate 549, the KPC-3-producing isolate belonged to PFGE type U, with 79.8% similarity with type Q.

MLST of these isolates was concordant with the PFGE data. Four STs were identified: ST 340 (PFGE type R), ST 277 (PFGE type P), a novel ST designated ST 376 (PFGE type S) and ST 327 (PFGE type U). The epidemic PFGE type Q belonged to ST 258 as reported earlier (8) (Figure 1). ST 340 and ST 277 are genetically close variants of ST 258; ST 340 is a single locus variant (*tonB* allele) and ST 277 is a double locus variant (*infB* and *tonB* alleles) of ST 258. This close genetic relatedness may propose a common ancestor.

Except for the epidemic worldwide disseminated clone *K. pneumoniae* ST 258 the described KPC-producing clones differed from the previously reported KPC-producing isolates from the United States (8). Inspection of all the KPC-producing *K. pneumoniae* STs described in the literature including the present study indicates that the majority of the strains are scattered throughout the *K. pneumoniae* evolutionary tree rather than clustered into a specific genetic lineage (Brisse S, personal communication) suggesting that dissemination of KPC resistance is due to horizontal gene transfer rather than clonal spread. The only strains that revealed large degree of
genetic relatedness were ST 277, ST 258 and ST 340 proposing clonal relation between these strains.

**Antibiotic susceptibilities and the presence of resistance genes**

All the studied isolates were extremely drug resistant irrespective of ST-type. Carbapenems MICs of the KPC-2-producing isolates were similar to the MICs of the KPC-3-producing ST 258 strain. ST 258 is typically resistant to amikacin and susceptible to gentamicin (9) whereas in the KPC-2-producing strains resistances to amikacin and gentamicin vary (Table 1). Six of 10 KPC-producing isolates were ESBL producers. In contrast to the Israeli ST 258 that was reported as a non-ESBL-producer (10) ST-327 the other KPC-3-producing clone was an ESBL-producer and so were five of the eight KPC-2-producing isolates. Multiple β-lactamases were identified, which varied within the same ST-type and between different ST-types (Table 1). Plasmid-mediated quinolone resistance gene \[\text{aac}(6')\text{-Ib-cr}\] was detected only in *K. pneumoniae* ST 376 and ST 277 (3).

**KPC-encoding plasmids comparison and origin**

Carbapenem-resistant *K. pneumoniae* clones carried multiple plasmids. Transformation following with \(\text{bla}_{\text{KPC}}\)-carrying plasmid analysis showed different size plasmids ranging from 45-95 kb for the \(\text{bla}_{\text{KPC-2}}\)-carrying plasmids and a 105 kb for the \(\text{bla}_{\text{KPC-3}}\)-carrying plasmids (Figure 2A).

Acquisition of \(\text{bla}_{\text{KPC}}\)-carrying plasmids rendered resistance to piperacillin, cephalosporins, aztreonam. MICs of carbapenems increased from 0.094, 0.012, and 0.012 µg/ml for imipenem, meropenem and ertapenem to MIC\(_{50}\) of 8, 4 and 4 µg/ml respectively. MICs of quinolones remained similar and resistances to amikacin, gentamicin and trimethoprim/sulfamethoxasole varied between transformants (Table 1).
Southern blot analysis using a labeled \( \text{bla}_{\text{KPC}} \) probe proved the presence of a single KPC encoding plasmid in each of the \( K. \) \( \text{pneumoniae} \) strains. The migration pattern of KPC-2-encoding plasmids varied whereas for the two KPC-3-producing clones was similar (Figure 2B). Plasmid restriction analysis showed that \( \text{bla}_{\text{KPC-2}} \)-carrying plasmids differed between clones but were similar or identical within the same clone and they differed from the \( \text{bla}_{\text{KPC-3}} \)-carrying plasmid. The two KPC-3-producing \( K. \) \( \text{pneumoniae} \) strains ST 258 and ST 327 carried the same plasmid, pKpQIL, reported previously (10). Interestingly, \( K. \) \( \text{pneumoniae} \) ST 327 was isolated from a patient that was co-infected with \( K. \) \( \text{pneumoniae} \) ST 258. This suggests the possibility of the horizontal transfer of pKpQIL from ST 258 to ST 327 within this same patient.

The origin of the KPC-encoding plasmids is unclear. pKpQIL, the Israeli \( \text{bla}_{\text{KPC-3}} \)-carrying plasmid differs from \( \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{KPC-3}} \)-carrying plasmids of strains belonging to ST 258 from the United States (12). As for the \( \text{bla}_{\text{KPC-2}} \)-carrying plasmids, they may have been originated from other enteric pathogens. \( \text{bla}_{\text{KPC-2}} \) appeared initially among \( \text{Enterobacter} \) and \( E. \) \( \text{coli} \) isolates during 2004-2005 in our hospital (2, 6, 11). We may speculate that the gene originated from these organisms via horizontal transfer. \( \text{bla}_{\text{KPC-2}} \)-carrying plasmids derived from \( \text{Enterobacter} \) strains were significantly larger than the \( K. \) \( \text{pneumoniae} \) plasmids ~200Kb (2) and thus rules out the possibility that they had transferred to the latter organism. To verify the possibility of plasmid origin from \( E. \) \( \text{coli} \), all \( \text{Klebsiella} \) \( \text{bla}_{\text{KPC-2}} \)-carrying plasmids were compared to \( \text{bla}_{\text{KPC-2}} \)-carrying plasmids from seven carbapenem-resistant \( E. \) \( \text{coli} \) strains isolated during the same period in which the \( K. \) \( \text{pneumoniae} \) isolates were identified (6, 11). Plasmid comparison indicated that two of the \( \text{bla}_{\text{KPC-2}} \)-carrying plasmids of \( K. \) \( \text{pneumoniae} \) were identical to \( E. \) \( \text{coli} \) plasmids. The strains that shared
common plasmids were: *K. pneumoniae* ST 340 and *E. coli* strain 386 (plasmid size 50kb) (Figure 3 lanes 2-3, 6-7, 10-11); *K. pneumoniae* ST 376 and *E. coli* strain 547 (75 kb) (Figure 3 lanes 4-5, 8-9, 12-13). These data suggests the horizontal transfer of the intact blaKPC-2-carrying plasmid between these two species (Figure 3). Based on restriction analysis *K. pneumoniae* ST 277 did not show similarity with any of the *E. coli* plasmids suggesting the possibility of acquisition of blaKPC-2 through Tn4401 transposition between plasmids followed by their independent horizontal transfer (13), or plasmid rearrangement that may have affected the restriction pattern obtained.

Carbapenem-resistant KPC-producing *K. pneumoniae* is spreading worldwide posing a real threat (13). Characterizing the strains and plasmids involved, may aid in understanding the evolution and thereby control of the dissemination of this clinically important antibiotic resistant phenotype. In this current study, we characterized all the KPC-2-harboring *K. pneumoniae* isolates from our hospital by MLST, plasmid mapping and comparisons. We demonstrated a complex epidemiology that involves limited clonal and plasmid transmission via horizontal transfer either within the same species, like the blaKPC-3-carrying plasmid, or via rare events of intra-species transmission, like the blaKPC-2-carrying plasmid from *E. coli.*
Acknowledgements

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We would like to sincerely thank the Klebsiella pneumoniae MLST website researchers, particularly Dr. Sylvain Brisse, for their professional assistance and for providing the evolutionary tree of K. pneumoniae.

We also thank Daphne Karfunkel for the critical reading of this manuscript.
References


## Table 1 Antibiotic susceptibilities, STs, bla<sub>KPC</sub> alleles and bla genes of the studied *K. pneumoniae* isolates

<table>
<thead>
<tr>
<th>K. pneumoniae isolates (ST-type) and transformants (T)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>bla genes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MIC (µg/ml)</th>
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<tr>
<td></td>
<td>AMK</td>
<td>GEN</td>
</tr>
<tr>
<td>KPC-2 producers</td>
<td></td>
<td></td>
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<tr>
<td>475 (ST 277)</td>
<td>SHV-27, CTX-M-15, OXA-4&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
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<td>4</td>
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<tr>
<td>469-T</td>
<td>16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>523 (ST 340)</td>
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<td>16</td>
</tr>
<tr>
<td>523-T</td>
<td>&gt;64</td>
<td>2</td>
</tr>
<tr>
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<td>32</td>
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<tr>
<td>365 (ST 376)</td>
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<td>4</td>
</tr>
<tr>
<td>365-T</td>
<td>&lt;2</td>
<td>&lt;1</td>
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</table>

<sup>a</sup> Transformants (T) are derived from the parent strains by homologous recombination.

<sup>b</sup> bla genes: AMK, GEN, CAZ, CRO, ATM, TZP, IPM<sup>c</sup>, MEM<sup>c</sup>, ERT<sup>c</sup>, CIP, LVX, CST<sup>d</sup>, SXT, TGC<sup>d</sup>.

<sup>c</sup> MIC values for β-lactam antibiotics: AMK, GEN, CAZ, CRO, ATM, TZP, IPM, MEM, ERT, CIP, LVX, CST, SXT.

<sup>d</sup> MIC values for β-lactam antibiotics: AMK, GEN, CAZ, CRO, ATM, TZP, IPM, MEM, ERT, CIP, LVX, CST, SXT, TGC.

<sup>f</sup> KPC-2 producers.
<table>
<thead>
<tr>
<th>531 (ST 376)</th>
<th>SHV-1</th>
<th>8</th>
<th>&lt;1</th>
<th>&gt;64</th>
<th>&gt;64</th>
<th>&gt;128</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>&gt;4</th>
<th>&gt;8</th>
<th>0.125</th>
<th>&lt;20</th>
<th>1.5</th>
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<td>16</td>
<td>64</td>
<td>64</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>0.032</td>
<td>&lt;20</td>
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<td>&gt;128</td>
<td>32</td>
<td>64</td>
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<td>&gt;4</td>
<td>&gt;8</td>
<td>0.125</td>
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<td>1</td>
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<tr>
<td>525-T</td>
<td>16</td>
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<td>16</td>
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<tr>
<td>549 (ST 327)</td>
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<td>&gt;16</td>
<td>16</td>
<td>&gt;64</td>
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<tr>
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<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;128</td>
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<td>557-T</td>
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<td>&lt;0.25</td>
<td>0.032</td>
<td>&lt;20</td>
<td>0.125</td>
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| *E. coli* Genehogs | <2 | <1 | <1 | <1 | <4 | 0.094 | 0.012 | 0.012 | 0.002 | 0.006 | 0.125 | <20 | 0.094 |

a Transformant were in E. coli Genehogs.

b Identified using ESBL confirmatory test (CLSI) and PCR

c Identified using agar dilution

d Identified using Etest
Figure legends

Figure 1
The genetic relatedness of blaKPC-2 and blaKPC-3-producing carbapenem-resistant K. pneumoniae clones in Tel Aviv Medical Center during the years 2005-2006. blaKPC alleles, sequence types and the date of isolation is presented in the right.

Figure 2
Plasmid analysis of ten blaKPC-2 and blaKPC-3 carrying carbapenem-resistant K. pneumoniae clinical strains (lanes D) and their respective transformants (T), C= chromosomal DNA, panel A; Southern blot analysis of plasmid DNA of K. pneumoniae isolates, clinical strains (D) and their respective transformants (T), panel B; The restriction pattern of plasmid DNA derived from K. pneumoniae transformants digested with BglII (lanes 1-10) and SmaI (lanes 11-20), Panel C. PFGE types defined in figure 1 are presented in the upper part of the Figure.

Figure 3
A comparison of blaKPC-2-carrying plasmids of plasmids originated from two K. pneumoniae and two E. coli clones isolated in the same time period. Plasmid restriction analysis of transformants carrying these plasmids showed identity between the K. pneumoniae plasmids (K) and the E. coli plasmids (E). Plasmids from both organisms were digested with BglII (lanes 2-5), EcoRV (lanes 6-9) and SmaI (lanes10-13) prior to electrophoresis. GeneRuler 1-Kb DNA ladder (Fermentas Life Sciences), Lane 1 (M); E. coli 386 (lanes 2, 6, 10); K. pneumoniae 523 PFGE type R (lanes 3, 7, 11); E.coli 547 (lanes 4, 8, 12) and K. pneumoniae 531, PFGE type (lanes 5, 9, 13).