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

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Received 23 December 2009/Returned for modification 14 February 2010/Accepted 20 March 2010

Sporadic isolates of carbapenem-resistant KPC-2-producing *Klebsiella pneumoniae* were isolated in Tel Aviv Medical Center during 2005 and 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 (8 KPC-2 and 2 KPC-3 producing) were studied. All isolates were extremely drug resistant. They possessed the *bla*KPC gene and varied in their additional beta-lactamase contents. 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 to 95 kb) and differed among 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 indicate 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.

KPC-producing carbapenem-resistant *Klebsiella pneumoniae* strains have increasingly been reported worldwide. The first KPC-producing *K. pneumoniae* isolate identified in Tel Aviv Medical Center, Tel Aviv, Israel, 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, sequence type (ST) 258 (pulsed-field gel electrophoresis [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 Israeli KPC-producing *K. pneumoniae* strains have been 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*KPC alleles. In the United States, for example, isolates belonging to ST 258 possess both *bla*KPC-2 and *bla*KPC-3 (8). Other places report the existence of a single allele carried by this clone, such as *bla*KPC-2 in Norway and Greece (15), Poland (1), and Finland (14) and *bla*KPC-3 in the United Kingdom (18), Sweden (15), and Italy (5).

Molecular studies of the *bla*KPC-2-carrying plasmid of *K. pneumoniae* ST 258 in Israel showed that it harbored a 105-kb *bla*KPC-3-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 coexistence in our hospital of KPC-2-carrying *K. pneumoniae* isolates and KPC-3-carrying *K. pneumoniae* ST 258 isolates led to this study. We aimed to investigate the molecular epidemiology of these strains, examine their evolutionary relatedness, compare their *bla*KPC-carrying plasmids, and facilitate the understanding of the origin of these plasmids in *K. pneumoniae* by comparing them to *bla*KPC-2-carrying plasmids from other *Enterobacteriaceae* in our hospital, such as KPC-producing *Escherichia coli*.

(This work was performed by Azita Leavitt in partial fulfillment of the requirements for a Ph.D. from the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.)

(This study was presented in part at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy [ICAAC], San Francisco, CA, 2009, abstr. C2-667.)

MATERIALS AND METHODS

Study isolates. Eight carbapenem-resistant KPC-2-producing *K. pneumoniae* clinical isolates that comprise the entire collection of KPC-2 isolates isolated in the clinical laboratory of Tel Aviv Medical Center during 2005 and 2006 were studied; all of them showed non-Q pulsortypes (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 (CLSI) (4). Susceptibility testing for colistin and tigecycline was per-
FIG. 1. The genetic relatedness of bla_{KPC-2} and bla_{KPC-3}-producing carbapenem-resistant *K. pneumoniae* clones in Tel Aviv Medical Center during the years 2005 and 2006. *bla*_{KPC}-alleles, sequence types, and the date of isolation are presented on the right. Isolates were clustered into five different clusters based on GelComap Dic algorithm coefficients, which range from 0% to 100%, and using a tolerance of 1.5%, as illustrated by the scale to the left of each dendrogram. Two of the three ST 340 isolates were further analyzed.

formed by Etest according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). The 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 U.S. 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, Smal, and EcoRV (New England Biolabs, Boston, MA), and their restriction patterns were 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. Plasmid sizes were determined using ST treatment following PFGE (10) and compared using GelComap II software.

**RESULTS AND DISCUSSION**

**Epidemiology and genetic relatedness of carbapenem-resistant *K. pneumoniae* isolates.** Genotyping of all the KPC-2-producing isolates revealed that they were genetically different (<85% identity) from KPC-3-producing ST 258 (PFGE type O). 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', respectively (Fig. 1). Isolate 549, the KPC-3-producing isolate, belonged to PFGE type U, with 79.8% similarity with type Q. MLST data for these isolates were concordant with the PFGE data. Four STs were identified: ST 340 (PFGE type R), ST 277 (PFGE type P), a novel ST designated ST 377 (PFGE type S), and ST 327 (PFGE type U). The epidemic PFGE type Q belonged to ST 258 as reported earlier (8) (Fig. 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 support the proposal of 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 (S. Brisse, personal communication), suggesting that dissemination of KPC resistance is due to horizontal gene transfer rather than clonal spread. The only strains that revealed a large degree of genetic relatedness were ST 277, ST 258, and ST 340, supporting the proposal of clonal relation between these strains.

**Antibiotic susceptibilities and the presence of resistance genes.** All isolates studied were extremely drug resistant irrespective of ST type. Carbapenem 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 ten KPC-producing isolates were extended-spectrum beta-lactamase (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 and varied within the same ST type and between different ST types (Table 1). The plasmid-mediated quinolone resistance gene (*aac(6’)-Ib-cr*) was detected only in *K. pneumoniae* ST 376 and ST 277 (3).

**KPC-encoding plasmid comparison and origin.** Carbapenem-resistant *K. pneumoniae* clones carried multiple plasmids.
TABLE 1. Antibiotic susceptibilities, STs, blaKPC alleles, and bla genes of the studied K. pneumoniae isolates

<table>
<thead>
<tr>
<th>K. pneumoniae isolate (ST type) or transformant</th>
<th>bla gene(s)</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPC-2 producers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>475 (ST 277) SHV-27, CTX-M-15, OXA-4</td>
<td>&lt;2 &gt;16</td>
<td>&lt;2 &gt;16 &gt;64</td>
</tr>
<tr>
<td>475-T</td>
<td>&gt;128 32 64</td>
<td>0.5 0.19</td>
</tr>
<tr>
<td>469 (ST 277) SHV-27, CTX-M-15, OXA-4</td>
<td>4 &gt;16 &gt;64</td>
<td>&lt;0.25 &gt;0.12</td>
</tr>
<tr>
<td>469-T</td>
<td>&gt;128 32 64</td>
<td>0.25 0.125</td>
</tr>
<tr>
<td>523 (ST 340) TEM-1, SHV-11, OXA-2</td>
<td>16 &gt;16 &gt;64</td>
<td>&gt;0.25 0.25</td>
</tr>
<tr>
<td>523-T</td>
<td>&gt;128 32 64</td>
<td>0.047 0.2</td>
</tr>
<tr>
<td>588 (ST 340) TEM-1, SHV-11</td>
<td>32 &lt;1 &gt;16</td>
<td>0.19 &lt;0.2</td>
</tr>
<tr>
<td>588-T</td>
<td>&gt;128 32 64</td>
<td>0.047 0.2</td>
</tr>
<tr>
<td>365 (ST 376) SHV-1, CTX-M-15, OXA-4</td>
<td>4 &gt;16 &gt;64</td>
<td>&gt;0.125 &gt;0.125</td>
</tr>
<tr>
<td>365-T</td>
<td>&lt;2 &lt;1 &gt;16</td>
<td>&lt;0.25 &gt;0.125</td>
</tr>
<tr>
<td>531 (ST 376) SHV-1</td>
<td>32 &lt;1 &gt;64</td>
<td>0.125 &lt;2.0</td>
</tr>
<tr>
<td>531-T</td>
<td>&gt;128 32 64</td>
<td>0.125 &lt;2.0</td>
</tr>
<tr>
<td>525 (ST 376) SHV-1</td>
<td>8 &lt;1 &gt;64</td>
<td>&gt;0.25 &gt;0.25</td>
</tr>
<tr>
<td>525-T</td>
<td>&gt;128 32 64</td>
<td>0.032 &gt;0.1</td>
</tr>
<tr>
<td>526 (ST 376) SHV-1</td>
<td>4 &lt;1 &gt;64</td>
<td>&gt;0.125 &gt;0.125</td>
</tr>
<tr>
<td>526-T</td>
<td>&lt;2 &lt;1 &gt;16</td>
<td>&lt;0.25 &gt;0.125</td>
</tr>
<tr>
<td>KPC-3 producers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>549 (ST 327) TEM-1, CTX-M-2</td>
<td>&lt;2 &gt;16</td>
<td>&lt;2 &gt;16 &gt;64</td>
</tr>
<tr>
<td>549-T</td>
<td>&gt;320 0.75</td>
<td>&gt;8 0.125</td>
</tr>
<tr>
<td>557 (ST 258) TEM-1</td>
<td>32 &lt;1 &gt;64</td>
<td>&lt;0.25 &gt;0.25</td>
</tr>
<tr>
<td>557-T</td>
<td>&gt;320 128 256</td>
<td>&gt;8 0.125</td>
</tr>
<tr>
<td>E. coli Genehogs</td>
<td>&lt;2 &lt;1 &lt;1</td>
<td>0.094 0.012</td>
</tr>
</tbody>
</table>

Plasmid analysis of transformants showed different-sized plasmids ranging from 45 to 95 kb for the blaKPC-2-carrying plasmids and 105 kb for the blaKPC-3-carrying plasmids (Fig. 2A).

Acquisition of blaKPC-carrying plasmids rendered resistance to piperacillin, cephalosporins, and aztreonam. MICs of carbapenems increased from 0.094, 0.012, and 0.012 µg/ml for imipenem, meropenem, and ertapenem to MICₘ₉₀ of 8, 4, and 4 µg/ml, respectively. MICs of quinolones remained similar, and resistances to amikacin, gentamicin, and trimethoprim-sulfamethoxazole varied between transformants (Table 1).

Southern blot analysis using a labeled blaKPC probe proved the presence of a single KPC-encoding plasmid in each of the K. pneumoniae strains. The migration pattern of KPC-2-encoding plasmids varied, whereas for the two KPC-3-producing clones, the patterns were similar (Fig. 2B). Plasmid restriction analysis showed that blaKPC-2-carrying plasmids differed between clones but were similar or identical within the same clone and that they differed from the blaKPC-3-carrying plasmid. The two KPC-3-producing K. pneumoniae strains ST 258 and ST 327 carried the same plasmid, pKPQIL, reported previously (10). Interestingly, K. pneumoniae ST 327 was isolated from a patient that was coinfect with K. pneumoniae ST 258. This suggests the possibility of the horizontal transfer of pKPQIL from ST 258 to ST 327 within this patient. The origin of the KPC-encoding plasmids is unclear. pKPQIL, the Israeli blaKPC-3-carrying plasmid differs from blaKPC-2 and blaKPC-2-carrying plasmids of strains belonging to ST 258 from the United States (12). As for the blaKPC-2-carrying plasmids, they may have originated from other enteric pathogens. blaKPC-2 appeared initially among Enterobacter and E. coli isolates during 2004 and 2005 in our hospital (2, 6, 11). We may speculate that the gene originated from these organisms via horizontal transfer. blaKPC-2-carrying plasmids derived from Enterobacter strains (sized ~200 kb) were significantly larger than the K. pneumoniae plasmids (2) and thus rule out the possibility that they had transferred to the
latter organism. To verify the possibility of plasmid origin from *E. coli*, all *Klebsiella bla* KPC-2-carrying plasmids were compared to *bla* KPC-2-carrying plasmids from seven carbapenem-resistant *E. coli* strains isolated during the same period in which the *K. pneumoniae* isolates were identified (6, 11). Plasmid comparison indicated that two of the *bla* KPC-2-carrying plasmids of *K. pneumoniae* were identical to *E. coli* plasmids. The strains that shared common plasmids were *K. pneumoniae* ST 340 and *E. coli* strain 386 (plasmid size, 50 kb) (Fig. 3, lanes 2, 3, 6, 7, 10, and 11), *K. pneumoniae* ST 376, and *E. coli* 547 (75 kb) (Fig. 3, lanes 4, 5, 8, 9, 12, and 13). These data suggest the horizontal transfer of the intact *bla*KPC-2-carrying plasmid between these two species (Fig. 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 *bla*KPC-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 the 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 *bla*KPC-3-carrying plasmid, or via rare events of intraspecies transmission, like the *bla*KPC-2-carrying plasmid from *E. coli*.

**ACKNOWLEDGMENTS**

This work was supported in part by the European Commission Research grant FP7: SATURN—Impact of Specific Antibiotic Therapies on the Prevalence of Human Host Resistant Bacteria (grant no. 241796).

We would like to sincerely thank the *Klebsiella pneumoniae* MLST website researchers, particularly 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 the manuscript.
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