Detection of aac(6′)-Ib-cr in KPC-producing Klebsiella pneumoniae isolates from Tel Aviv, Israel

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Objectives: We aimed to evaluate the occurrence and characteristics of plasmid-mediated quinolone resistance (PMQR) genes in KPC-producing (KPC-P) Klebsiella pneumoniae (Kpn) isolates in Tel Aviv Medical Center, Israel.

Methods: Forty-seven KPC-P Kpn isolates were studied. Antibiotic susceptibilities were determined by Vitek 2, Etest or agar dilution. β-Lactamases and PMQR determinants were detected by PCR. For plasmid characterization, transformation, transconjugation, restriction mapping and Southern blot analysis were performed.

Results: Six out of 47 (13%) KPC-P isolates carried aac(6′)-Ib-cr. Acquisition of aac(6′)-Ib-cr-encoding plasmids increased the MIC of ciprofloxacin by 2-fold. In five of six KPC-P isolates, aac(6′)-Ib-cr and blaKPC-2 were encoded on the same plasmid.

Conclusions: The most prevalent PMQR gene in the studied KPC-P K. pneumoniae isolates is aac(6′)-Ib-cr. The co-existence of PMQR genes with KPC on the same plasmid poses a serious epidemiological, clinical and public-health threat.

Keywords: β-lactamases, carbapenem resistance, quinolone resistance, plasmids

Introduction

Multiple plasmid-mediated quinolone resistance (PMQR) determinants, including the five qnr genes qnrA, qnrB, qnrS, qnrC and qnrD,1–5 the aminoglycoside acetyltransferase-encoding enzyme variant aac(6′)-Ib-cr6 and the efflux-pump-encoding qepA7, are all involved in resistance to fluoroquinolones. These genes confer low-level quinolone resistance that can facilitate the selection of chromosomal mutations and high-level resistance.8

With the increasing prevalence of plasmid-encoded Klebsiella pneumoniae carbapenemase (KPC) in various parts of the world, PMQR genes were sought on the same plasmids. The co-existence of qnrA and qnrB with blaKPC has been reported in K. pneumoniae from China and the USA.9 We recently reported the co-existence of qnrB2 and blaKPC-2 on a single plasmid in Enterobacter cloacae from Israel. One of these isolates also carried aac(6′)-Ib-cr on a separate plasmid.10

Approximately 6% of the K. pneumoniae isolates in the Tel Aviv Medical Center carry KPC (S. Navon-Venezia, M. Schwaber and Y. Carmeli, unpublished results). We aimed to evaluate systematically the occurrence of the PMQR determinants qnr and aac(6′)-Ib-cr among KPC-producing K. pneumoniae clinical isolates in the Tel Aviv Medical Center.

Materials and methods

Bacterial strains, susceptibility and genetic relatedness

Forty-seven KPC-producing (KPC-P) K. pneumoniae single patient isolates collected in Tel Aviv Medical Center, a 1200 bed tertiary care teaching hospital, between the years 2004 and 2006 were studied. Isolates were molecularly characterized and antibiotic susceptibilities were determined by Vitek 2 (BioMerieux Inc., Marcy, France). MICs of carbapenems, and ciprofloxacin and levofloxacin were determined via Etest (AB Biodisk, Solna, Sweden), and of nalidixic acid by agar dilution (CLSI guidelines11). Genetic relatedness was determined using PFGE after SpeI restriction. PFGE DNA macrorestriction patterns were compared using GelComparII software (Applied Maths, Belgium).
**PMQR and β-lactamase screening**

PCR using primers specific for *bla*KPC (F: ATGTCACTGTATGCGC GTCT; R: TTTCAGACGGTTACTGTCCCC) followed by sequencing was performed. Screening for *qnrA*, *qnrB* and *qnrS* was carried out using multiplex PCR with primers described by Robicsek et al. Screening with primers specific for *qnrC* (F: GGGTTGTAC ATTTAATTGACAG; R: CACCTACCATTAATTATC), *qnrD* and *qepA* (F: AACTGCTGAGCCCGTAGAT; R: GTCGACCTGCCATGCA) was performed. The presence of *aac(6’)-Ib-cr* was determined by PCR followed by BtsCI digestion and sequencing. Gene fragments surrounding the quinolone resistance-determining region in *gyrA* and *parC* were amplified using primers *gyrA*6 and *gyrA*613R (F: CGA CCTTGCGAGAAAT; R: GTTCCATACGGCCTTCA), and *parC91* and *parC583R* (F: TACGTCACTGAGACAG; R: GCCATTCACTCGGTTG), respectively, and sequenced. Screening for the presence of extended-spectrum β-lactamase (ESBL) genes in PMQR-positive isolates was carried out. Amplification using primers recognizing the *bla*SHV-group (F: TTTATCGCCYCTACT CAAGG; R: GCTGCGGCCCAGATAACG) and the *bla*TEM-group (F: KACAATATACCTGGTAATGC; R: AGTATATATGAGT AACCTTGG) genes was performed. Obtained amplicons were sequenced. PCR with primers specific for *blacTX-M-3* (F: ATGTA TGACTACAGACATTG; R: TTATGTCAGACAAAGCTTG), *blacTX-M-25* (F: CACACAATTGATGAAGTTCCAG; R: TCACTCCACA TGTTAGAT) and *blacTX-M-9* (F: GTGACAAAGAGAGATGCAC CGG; R: ATGTTCCTCGCGGATAGCC) groups was performed. The *blacTX-M-3* group genes were amplified using the primers *CTX-M-3FF* (GTTGTTGTTATTTCGTATCTTCC) and *CTX-M-3FR* (CGATAAACAAAACCGGAATG), and sequenced.

**Plasmid analysis and transfer of PMQR genes**

Plasmids were isolated using the NucleoBond PC 100 midi kit (Macherey-Nagel, Düren, Germany). Transformation was performed by electroporation (Electroporator 2510, Eppendorf, Hamburg, Germany) into *Escherichia coli* strain DH10B (Invitrogen, Carlsbad, CA, USA). Transformants were selected on LB agar plates containing 100 mg/L ampicillin. Transformed colonies were screened for the presence of *aac(6’)-Ib-cr* via PCR followed by BtsCI digestion. The presence of *blacTX-M-15, blacKPC* and *bla*SHV in the transformed colonies was verified by PCR. Size estimation of transformed plasmids was carried out by treatment with S1 nuclease (Promega, Madison, WI, USA), followed by PFGE as described previously. The Lambda Ladder PFGE marker (New England Biolabs, Ipswich, MA, USA) was used as a molecular size marker.

Transconjugation experiments were performed with isolate 475, using the filter-mating method. The rifampicin-resistant *K. pneumoniae* strain 29RR was used as a recipient strain. 475RR was successful. The MIC of ciprofloxacin for the transconjugant increased 2.6-fold (from 0.38 to 1 mg/L), relative to the recipient clone 29RR. The transconjugant carried a plasmid with *aac(6’)-Ib-cr* and *bla*KPC-2. The *bla*SHV-27 gene was not transferred by transconjugation.

**Results**

**Prevalence of PMQR determinants and antibiotic susceptibilities**

Forty-seven KPC-*K. pneumoniae* isolates were characterized, of which 43 (91%) were resistant to quinolones. Two isolates were resistant to levofloxacin and had an intermediate ciprofloxacin MIC (2 mg/L).

The *qnr* groups and the *qepA* gene were not detected via PCR screening. PCR followed by digestion with BtsCI showed the restriction pattern of an *aac(6’)-Ib-cr* variant in six of the isolates (12.8%). Sequencing confirmed the presence of *aac(6’)-Ib-cr* by revealing two known mutations (W102R and D179Y) in all six isolates. These *aac(6’)-Ib-cr*-carrying isolates belonged to three different genetic clones. Two isolates (belonging to clone P) were susceptible to quinolones and four isolates (belonging to clones B and S) were resistant. Susceptibility data for the other antimicrobial agents are presented in Table 1.

All KPC-producing *aac(6’)-Ib-cr*-carrying isolates carried *bla*KPC-2. Two strains, belonging to two different clones (B and P), also carried *bla*CTX-M-15. All isolates belonging to clones B and S carried *bla*SHV-1; isolates 469 and 475 (clone P) carried *bla*SHV-27. Genes belonging to the *bla*TEM family and to *bla*CTX-M groups other than *bla*CTX-M-15 were not detected via PCR screening. All four quinolone-resistant isolates (clones B and S) had, in addition to PMQR genes, two mutations in *gyrA* and one mutation in *parC* (Table 1).

**Plasmid analysis**

Transconjugation of the *aac(6’)-Ib-cr*-carrying plasmid derived from *K. pneumoniae* isolate 475 was successful. The MIC of ciprofloxacin for the transconjugant increased 2.6-fold (from 0.38 to 1 mg/L), relative to the recipient clone 29RR. The transconjugant carried a plasmid with *aac(6’)-Ib-cr* and *bla*KPC-2. The *bla*SHV-27 gene was not transferred by transconjugation.

Transformation experiments succeeded for all KPC-producing isolates, except for *K. pneumoniae* isolate 475. MICs of ciprofloxacin in the transformants increased by 1.5- to 2-fold as compared with the *E. coli* DH10B recipient strain, whereas MICs of levofloxacin did not change upon plasmid acquisition, as expected. MICs of carbapenems increased in most of the transformants, although they did not reach the same MICs as those of the donors (Table 1).

PCR analysis of transformants revealed that in isolates belonging to clone S, *aac(6’)-Ib-cr*-co-existed with *bla*KPC-2 on the same plasmid. In isolate 469 (clone P), *aac(6’)-Ib-cr*-co-existed with *bla*KPC-2 and *bla*CTX-M-15 on the same plasmid. In clone B, isolate *aac(6’)-Ib-cr*-co-existed with *bla*CTX-M-15 on the same plasmid, whereas *bla*KPC-2 was carried on a different plasmid. The *bla*SHV genes were not transferable (Table 1).

The three isolates derived from clone S carried *aac(6’)-Ib-cr*-encoding plasmids similar in size (migrated between lambda marker bands 48.5–97 kb) (Figure 1a and b, lanes 525 D,T, 526 D,T and 531 D,T), but different in size from *aac(6’)-Ib-cr*-encoding plasmids of clone B (~48.5 kb) (Figure 1a and b, lanes 365 D,T,11) and of clone P (~97 kb) (Figure 1a and b, lanes 469 D,T).

Southern blot analysis of donors and transformants hybridized with *aac(6’)-Ib-cr* or *bla*KPC-2 probes confirmed that...
Table 1. PFGE, resistance genes and antibiotic susceptibilities of the six PMQR-carrying KPC-producing *K. pneumoniae* (Kpn) clinical isolates and their transformants

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>PFGE</th>
<th>PMQR</th>
<th>gyrA</th>
<th>parC</th>
<th>bla genes</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NAL</td>
</tr>
<tr>
<td>Kpn 365</td>
<td>B</td>
<td>aac(6′)-Ib-cr</td>
<td>S83F D87N S80I</td>
<td>KPC-2, SHV-1, CTX-M-15</td>
<td>&gt;256</td>
<td>&gt;32</td>
</tr>
<tr>
<td>365-Ta1</td>
<td>aac(6′)-Ib-cr</td>
<td>CTX-M-15</td>
<td>KPC-2</td>
<td>2</td>
<td>0.004</td>
<td>0.006</td>
</tr>
<tr>
<td>365-Ta2</td>
<td>aac(6′)-Ib-cr</td>
<td>KPC-2</td>
<td>2</td>
<td>0.002</td>
<td>0.006</td>
<td>≤2</td>
</tr>
<tr>
<td>Kpn 525</td>
<td>S</td>
<td>aac(6′)-Ib-cr</td>
<td>S83F D87N S80I</td>
<td>KPC-2, SHV-1</td>
<td>&gt;256</td>
<td>&gt;32</td>
</tr>
<tr>
<td>525-T</td>
<td>aac(6′)-Ib-cr</td>
<td>KPC-2</td>
<td>2</td>
<td>0.004</td>
<td>0.006</td>
<td>16</td>
</tr>
<tr>
<td>Kpn 526</td>
<td>S</td>
<td>aac(6′)-Ib-cr</td>
<td>S83F D87N S80I</td>
<td>KPC-2, SHV-1</td>
<td>&gt;256</td>
<td>&gt;32</td>
</tr>
<tr>
<td>526-T</td>
<td>aac(6′)-Ib-cr</td>
<td>KPC-2</td>
<td>2</td>
<td>0.004</td>
<td>0.004</td>
<td>16</td>
</tr>
<tr>
<td>Kpn 531</td>
<td>S</td>
<td>aac(6′)-Ib-cr</td>
<td>S83F D87N S80I</td>
<td>KPC-2, SHV-1</td>
<td>&gt;256</td>
<td>&gt;32</td>
</tr>
<tr>
<td>531-T</td>
<td>aac(6′)-Ib-cr</td>
<td>KPC-2</td>
<td>2</td>
<td>0.003</td>
<td>0.006</td>
<td>16</td>
</tr>
<tr>
<td>Kpn 469</td>
<td>P</td>
<td>aac(6′)-Ib-cr</td>
<td>no mutations detected</td>
<td>KPC-2, SHV-27, CTX-M-15</td>
<td>4</td>
<td>0.064</td>
</tr>
<tr>
<td>469-T</td>
<td>aac(6′)-Ib-cr</td>
<td>KPC-2, CTX-M-15</td>
<td>2</td>
<td>0.004</td>
<td>0.006</td>
<td>8</td>
</tr>
<tr>
<td>Recipient DH10B</td>
<td>aac(6′)-Ib-cr</td>
<td></td>
<td>2</td>
<td>0.002</td>
<td>0.006</td>
<td>≤2</td>
</tr>
<tr>
<td>Kpn 475</td>
<td>P</td>
<td>aac(6′)-Ib-cr</td>
<td>no mutations detected</td>
<td>KPC-2, SHV-27</td>
<td>32</td>
<td>0.38</td>
</tr>
<tr>
<td>475-Ta1</td>
<td>aac(6′)-Ib-cr</td>
<td>KPC-2</td>
<td>256</td>
<td>1</td>
<td>0.38</td>
<td>16</td>
</tr>
<tr>
<td>475-Ta2</td>
<td>aac(6′)-Ib-cr</td>
<td>KPC-2</td>
<td>256</td>
<td>0.38</td>
<td>0.25</td>
<td>≤2</td>
</tr>
</tbody>
</table>

NAL, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; ATM, aztreonam; IPM, imipenem; MEM, meropenem; CRO, ceftriaxone; FEP, cefepime; CAZ, ceftazidime.

aTransformant.
bTransconjugant.
aac(6′)-Ib-cr and blaKPC-2 were encoded on different plasmids in clone B, and on the same plasmid in clones S and P (Figure 1b and c). In clone S, two additional plasmids hybridized with the aac(6′)-Ib-cr probe with lower intensity.

Discussion

The most prevalent PMQR gene among carbapenem-resistant K. pneumoniae isolates included in this study is aac(6′)-Ib-cr and was found in 6/47 (13%) of all isolates tested. In most isolates (5/6) it is co-carried with the blaKPC-2 gene on the same plasmid. Among the PMQR-carrying isolates, the blaCTX-M-15 gene is the most prevalent ESBL gene.

The aac(6′)-Ib-cr gene is involved in resistance to fluoroquinolones as well as to aminoglycosides.6 The gene has been detected in various locations, including the USA, Canada, Argentina, Portugal, France, China and Spain.9 Its prevalence varies between species; it has been reported to be more prevalent in E. coli than in K. pneumoniae or Enterobacter spp.

The presence of PMQR determinants, aac(6′)-Ib-cr and qnr, together with the KPC gene among nosocomial pathogens, is of great importance as it further limits the usefulness of quinolones in the treatment of these extremely drug-resistant strains. The co-existence of aac(6′)-Ib-cr with blactx-M-15, with or without blatem-1 and blaoxa-1, has been previously described in E. coli and K. pneumoniae strains.9 Here, we extended this finding to an even more difficult-to-treat pathogen, and found the co-existence of aac(6′)-Ib-cr and blakpc-2 on the same plasmid in two distinct clones of K. pneumoniae clinical isolates. The co-carriage provides an evolutionary benefit to these strains in an antibiotic-rich environment, and leads to their selection under both β-lactam and quinolone pressure. In this respect, aac(6′)-Ib-cr provides an even greater advantage by also conferring resistance to certain aminoglycosides.

The aac(6′)-Ib-cr-carrying plasmid was transconjugable. This provides an advantage in the dissemination of the aac(6′)-Ib-cr gene between clones, and even between species, which may contribute to its spread. For all these reasons, increasing awareness of the presence of PMQR determinants in clinical strains, and especially in extremely drug-resistant strains such as KPC-P Klebsiella strains, is necessary.

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Transparency declarations

None to declare.

References


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