Plasmid pKpQIL encoding KPC-3 and TEM-1 confers carbapenem resistance in an extremely drug-resistant epidemic Klebsiella pneumoniae strain

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Objectives: An extremely drug-resistant (XDR) clone of KPC-3-producing Klebsiella pneumoniae emerged in Israel in 2006, causing a nationwide outbreak. We aimed to characterize the local KPC-3-encoding plasmid carried by these isolates and study its contribution to antibiotic resistance.

Methods: Mechanisms of carbapenem resistance were investigated in seven selected isolates (isolated between 2006 and 2008) belonging to the epidemic clone. Isolates underwent MIC testing, and were examined for the presence of KPC, Tn4401, class I integron elements and additional antibiotic resistance genes. Plasmids were analysed by transformation, transconjugation, restriction mapping, curing and complementation experiments. Outer membrane protein (OMP) analysis was performed.

Results: OMP analysis did not reveal loss of porins. KPC-3-producing K. pneumoniae isolates possessed various plasmids but all harboured a common self-transmissible 105 kb plasmid, termed pKpQIL, encoding blaTEM-1 and blaKPC-3. Curing of pKpQIL led to a complete loss of resistance to cephalosporins and carbapenems, proving its crucial role in carbapenem resistance. Transformation of plasmid pKpQIL into the cured Klebsiella strain resulted in full reconstitution of carbapenem resistance. The presence of all Tn4401 transposon elements located upstream of the KPC-3 gene was detected by PCR and sequencing. pKpQIL lacked additional antibiotic resistance genes.

Conclusions: Our findings demonstrate the presence of pKpQIL, a 105 kb KPC-3- and TEM-1-encoding plasmid, in the XDR K. pneumoniae epidemic strain in Israel. pKpQIL is unique and appears consistently in all isolates of this clone over the years. The extensive β-lactam resistance phenotype of this clone is primarily mediated by this single self-transmissible plasmid.

Keywords: conjugation, curing, outer membrane proteins, carbapenem resistance

Introduction

Klebsiella pneumoniae is an important opportunistic pathogen and the causative bacteria of various nosocomially acquired infections. Carbapenem resistance in this species does not occur naturally and is mainly related to acquisition of carbapenem-hydrolysing β-lactamases of which the most common are the KPC type. These KPC-producing K. pneumoniae strains, originally isolated in the USA, have spread and recently are being increasingly detected with a wider geographical distribution including several countries in Europe, South America, China and the Middle East. In 2006, an extremely drug-resistant (XDR) K. pneumoniae genetically related to US strains from Arizona and New Jersey emerged throughout Israel, causing infections and leading to high morbidity and mortality.5 KPC-type carbapenemases are plasmid mediated and may be carried on various naturally occurring plasmids. The genetic environment of the KPC-2 gene derived from K. pneumoniae and Pseudomonas aeruginosa is organized on the Tn4401 transposon.5 Preliminary analysis of the blaKPC-3-carrying plasmid from multiple Israeli strains isolated during 2006–2008 revealed a common plasmid.5 We aimed to further characterize this plasmid and explore its role in conferring resistance among this epidemic K. pneumoniae clone.
Materials and methods

Bacterial strains

Seven carbapenem-resistant KPC-3-producing clinical isolates of K. pneumoniae belonging to the epidemic clone Q [identified as sequence type (ST) 258],7 isolated during 2006–2008, were included in this study. K. pneumoniae strain 557 (Kpn557) was used for curing experiments. K. pneumoniae strains 490 (Kpn490) and 557 (Kpn557) were used as the donor strains in conjugation experiments. Escherichia coli Genehogs (Invitrogen, UK) was used as a recipient strain in transformation experiments.

Bacterial identification, antibiotic susceptibility testing and PFGE

Bacterial identification and susceptibility testing were performed using Vitek 2 (bioMérieux, Marcy l’Étoile, France). MICs of carbapenems were determined by agar dilution and those of levofloxacin and ciprofloxacin by Etest according to the CLSI standard protocol.8 The extended-spectrum β-lactamase (ESBL) phenotype of the clinical Klebsiella strains was detected by the modified CLSI confirmatory test using boronic acid,9 and of the cured strain (Kpn557-C) by the standard ESBL confirmatory test.9 PFGE was used to verify the genetic identity of the cured K. pneumoniae strain 557 (Kpn557-C) with its parent strain.6

PCR for determination of antibiotic resistance genes and blaKPC genetic environment

Bacterial cell lysates from clinical strains, transformants and the cured isolate were used as templates in the specific PCR amplifications. PCR were performed using primers for blaKPC,10 ESBL genes11 and for characterization of the genes surrounding blaKPC. Additional primers were designed in this study for the resolvase tnpR gene (F: ACT GTG ACG CAT CCA ATG AG, R: ACC GAG GGA GAA TGG CTA CT) and for the insertion sequence ISKpn7 (F: GCA GGA TGA TTT CGT GGT CT, R: AGG AAG TCG GTG AAG CTG AA). Additional antibiotic resistance genes screened included aac(6’)-Ib, aminoglycoside resistance genes,12 class I integron elements,12 dhfr (F: GCC AAT CGG GTT ATT GGC AA, R: TGG GAA GAA GGC GTC ACC CTC) encoding trimethoprim/sulfamethoxazole resistance and plasmid-mediated quinolone-resistance determinants.11

The resulting PCR products were analysed and sequenced. The nucleotide acid and deduced protein sequences were analysed and compared by use of software available via the Internet at the NCBI website (http://www.ncbi.nlm.nih.gov/).

Outer membrane protein (OMP) analysis

OMPs were extracted13 from carbapenem-resistant isolates in low osmolality medium (nutrient broth) and high osmolality medium (Mueller–Hinton (MH) broth; Hy-Labs, Rehovot, Israel). K. pneumoniae ATCC strain 13883 was used as a control susceptible strain. After electrophoresis, protein bands of interest (possessing an apparent molecular weight of 35 and 36 kDa) were excised from the gel, and peptides were identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using an Ultimate™ nano HPLC system (LC Packings, Amsterdam, the Netherlands) and a Qstar Pulsar mass spectrometer (Applied Biosystems, Foster City, CA, USA). The MS data were analysed using the Mascot protein identification software (Matrix Science, London, UK).

Plasmid purification, restriction analysis and transformation

Plasmid DNA from the carbapenem-resistant K. pneumoniae isolates was obtained using the NucleoBond PC100 Macherey-Nagel (Germany) plasmid Midi kit. Plasmids isolated from clinical isolates and their transformants were digested with different restriction endonucleases such as BamHI and XhoI (New England Biolabs, Boston, MA, USA), and their restriction patterns were compared.

Plasmids were transformed by electroporation into the E. coli Genehogs strain. Transformants were selected on LB agar plates containing 100 mg/L ampicillin, and the resulting colonies were screened by PCR for the presence of blaKPC. Transformants possessing blaKPC were subjected to antibiotic susceptibility testing and further molecular characterization.

Conjugation experiments

Conjugation experiments were performed by filter mating,14 and bacterial suspensions were spread onto MH agar plates containing rifampicin (200 mg/L) and ertapenem (0.5 mg/L). Transconjugants were subjected to antibiotic susceptibility testing and PCR to determine the presence of blaKPC.

Detection and sizing of large plasmids by PFGE

PFGE following S1 nuclease treatment of genomic DNA embedded in agarose plugs was used to visualize high molecular weight plasmids in K. pneumoniae isolates.11

Curing experiments

Five carbapenem-resistant K. pneumoniae strains belonging to clone Q were subjected to elevated temperature-mediated plasmid elimination by sequential passages in brain heart infusion (BHI) broth at 42°C twice daily for a period of 2 weeks. After 2 weeks, cultures were diluted and plated on MH plates to obtain single colonies. Suspected cured colonies were identified by picking colonies and plating onto MH plates containing different concentrations of imipenem (ranging from 8 to 32 mg/L) and on a control MH plate. Colonies that failed to grow in the presence of imipenem were suspected to be cured and were further analysed for blaKPC loss by PCR, antibiotic susceptibility testing, plasmid profile and PFGE as mentioned above.

Complementation of plasmid DNA from Kpn557 into its cured form Kpn557-C was performed by transformation using electroporation and selection on LB agar plates containing 0.5 mg/L imipenem.

Results

Molecular mechanisms of antibiotic resistance

The seven XDR K. pneumoniae isolates belonging to the epidemic clone Q (ST258) were resistant to all cephalosporins, β-lactam/β-lactamase inhibitor combinations, trimethoprim/sulfamethoxazole, fluoroquinolones, amikacin and carbapenems. PCR and sequencing revealed the presence of blaKPC-3 and blaTEM-1 in all isolates studied.8 All isolates carried blaSHV-1 on their chromosomes. The clinical isolates did not produce ESBLs based on the CLSI boric acid confirmatory assay, and by negative molecular screening using PCR. We identified the presence of class I integron-related determinants including the intI1 gene, the conserved sequence 5CS 3CS, the dhfr gene encoding dihydrofolate reductase and the aac(6’)-Ib gene encoding aminoglycoside acetyltransferase.

OMP analysis performed on OMPs derived from clone Q isolates and from carbapenem-susceptible K. pneumoniae isolates, using SDS–PAGE followed by peptide identification by LC–MS/MS, revealed similar protein profiles including the two main OMPs,
OMPA (35 kDa) and OMPK36 (results are not shown), suggesting that carbapenem resistance in these isolates was not related to lack of OMPs.

**Analysis of plasmids and transfer of pKpQIL, the bla_{KPC-3}-encoding plasmid**

Plasmid analysis of the seven carbapenem-resistant *K. pneumoniae* isolates showed that they carried either two or three plasmids; six isolates carried three plasmids of 40, 105 and 200 kb (Figure 1a, lanes 1, 3, 7, 9, 11 and 13) and one isolate, Kpn557, carried two plasmids; a 105 kb plasmid and a larger plasmid of 240 kb (Figure 1a, lane 5). Plasmid analysis of the transformants (selected on ampicillin) following S1 treatment indicated acquisition of a 105 kb single plasmid (Figure 1a, lanes designated T). Acquisition of this plasmid by all transformants increased their MICs of extended-spectrum cephalosporins and aztreonam by >60-fold rendering them resistant. MICs of carbapenems increased significantly but not to full resistance, with an MIC_{50} of 2 mg/L for imipenem, and an MIC_{50} of 1 mg/L for meropenem and ertapenem. Restriction analysis of all bla_{KPC-3}-encoding plasmids isolated from the seven *E. coli* transformants using various DNA endonucleases revealed identical restriction patterns, proving identity between the plasmids (Figure 1b). We designated this KPC-3- and TEM-1-encoding plasmid, pKpQIL.

**Conjugation of bla_{KPC-3}-encoding plasmids**

The natural ability of pKpQIL to disseminate into other *Klebsiella* strains was examined by conjugation experiments using a rifampicin-resistant *K. pneumoniae* strain as a recipient. This strain was generated in this study by serial passages of *K. pneumoniae* strain 29 (kindly provided by Hanny Sahly, Hamburg, Germany) in rifampicin-containing BHI broth (50–800 mg/L) leading to a resistant strain, Kpn29RifR, with a rifampicin MIC >400 mg/L. Conjugation of the two clinical isolates Kpn490 and Kpn557 led to the isolation of two transconjugants harbouring plasmid pKpQIL: Kpn29-490RifR and Kpn29-557RifR, respectively (Table 1). Acquisition of pKpQIL conferred resistance to ceftazidime, aztreonam and the piperacillin/tazobactam combination, and increased MICs of imipenem from 0.25 to 4 mg/L, of meropenem from 0.25 to 1–2 mg/L, and of ertapenem from 0.012 to 1–1.5 mg/L. Resistance to aminoglycosides was not transferred and MICs of quinolones did not change (Table 1).

**Contribution of pKpQIL, the bla_{KPC-3}-carrying plasmid, to antibiotic resistance**

A curing procedure was attempted in five XDR *Klebsiella* isolates, but was successful in only one isolate; sequential passages of isolate Kpn557 (imipenem MIC 128 mg/L) resulted in growth of imipenem-susceptible colonies that lacked bla_{KPC} on PCR. The cured colonies possessed an identical PFGE DNA pattern, proving to be isogenic to their parent strain. The plasmid profile of the parent strain Kpn557 and its cured form, Kpn557-C, demonstrated a complete elimination of plasmid pKpQIL, the 105 kb plasmid carrying bla_{KPC-3}, leaving the parent strain with the larger sized 240 kb plasmid unique to this strain. Kpn557-C was susceptible to all cephalosporins, aztreonam and carbapenems; MICs of imipenem and meropenem decreased from 128 to 0.19 mg/L and the MIC of ertapenem decreased 128-fold from 256 to 2 mg/L. The MIC of ceftazidime decreased from 64 to 4 mg/L and that of piperacillin/tazobactam remained high (Table 1). Despite these high MICs, a phenotypic ESBL combination disc assay showed that the cured isolate lacking pKpQIL was not an ESBL producer. Curing of pKpQIL was associated with loss of bla_{TEM} in addition to bla_{KPC}, suggesting the co-existence of these two β-lactamases on the same plasmid. pKpQIL was successfully transformed into competent cured cells of Kpn557. The recovered colonies after transformation, Kpn557-R, were screened for the presence of bla_{KPC} by PCR. The plasmid pattern of the recovered strain that regained pKpQIL was identical to that of the Kpn557 parent strain. The carbapenem

![Figure 1](image-url)
Table 1. Antibiotic susceptibilities of the studied strains

<table>
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<th>MIC (mg/L)</th>
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<th>GEN</th>
<th>CAZ</th>
<th>CRO</th>
<th>ATM</th>
<th>TZP</th>
<th>IPM</th>
<th>MEM</th>
<th>ETP</th>
<th>CIP</th>
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<td>&gt;128</td>
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AMK, amikacin; GEN, gentamicin; CAZ, ceftazidime; CRO, ceftriaxone; ATM, aztreonam; TZP, piperacillin/tazobactam; IPM, imipenem; MEM, meropenem; ETP, ertapenem; CIP, ciprofloxacin; LVX, levofloxacin.

aTransformation was performed in E. coli Genehogs.
bTested by agar dilution.
cTested by Etest.

plasmid into strain, suggesting the potential of natural dissemination of this plasmid. MICs of ertapenem and ceftazidime decreased by 128- and 16-fold respectively. The MIC of piperacillin/tazobactam remained high (>128 mg/L). These relatively high MICs in a non-ESBL-producing background of the cured strain may be explained by non-β-lactamase-related modifications such as alteration in penicillin binding proteins. Curing caused loss of TEM-1 as well as the KPC-3 gene, suggesting the co-existence of these two genes on the same plasmid. Additional proof of the necessity of plasmid pKpQIL for carbapenem resistance was obtained by complete reconstitution of the carbapenem resistance phenotype by transformation of the plasmid back into the cured strain.

MICS of aminoglycosides and quinolones did not change after curing (Table 1), supporting our previous findings that showed that plasmid-mediated quinolone resistance determinants were not detected in Kpn557.11

Genetic environment of blaKPC-3

Analysis of the genetic elements surrounding blaKPC-3 located on pKpQIL using PCR and sequencing revealed the presence of all the genetic elements and insertion sequences belonging to Tn4401 described previously in KPC-2-producing K. pneumoniae and recently in KPC-3-producing strains from New York City. The KPC genetic environment of pKpQIL contained inverted repeat sequences, a resolvase gene (tnpR), a transposase gene (tnpA) and the insertion sequences ISKpn6 (ista) and ISKpn7 (istB). These elements were identified in all transformants carrying pKpQIL but were absent in Kpn557-C, the cured form lacking the plasmid.

Discussion

The emergence of an epidemic clone of K. pneumoniae resistant to carbapenems and to almost all existing antibiotics is of great clinical and public health importance. The widespread nature of this plasmid-mediated resistance in Israel led us to explore the KPC-3-encoding plasmid carried by this clone. An identical plasmid encoding KPC-3 of 105 kb (pKpQIL) was identified in all study isolates belonging to the highly epidemic clone during 2006–2008, and it was exclusively responsible for carbapenem resistance in these isolates.

pKpQIL was transferred successfully to a Klebsiella recipient strain, suggesting the potential of natural dissemination of this plasmid into Klebsiella strains leading to a carbapenem-resistant phenotype; this is the first report of transmission of a plasmid into a clinical Klebsiella strain.

Curing of the plasmid pKpQIL resulted in loss of resistance to cephalosporins and carbapenems. Exclusion of the plasmid decreased imipenem and meropenem MICS by 674-fold. The MICs of ertapenem and ceftazidime decreased by 128- and >16-fold, respectively, but remained elevated (2 and 4 mg/L, respectively). The MIC of piperacillin/tazobactam remained high (>128 mg/L). These relatively high MICs in a non-ESBL-producing background of the cured strain may be explained by non-β-lactamase-related modifications such as alteration in penicillin binding proteins. Curing caused loss of TEM-1 as well as the KPC-3 gene, suggesting the co-existence of these two genes on the same plasmid. Additional proof of the necessity of plasmid pKpQIL for carbapenem resistance was obtained by complete reconstitution of the carbapenem resistance phenotype by transformation of the plasmid back into the cured strain.
The Israeli carbapenem-resistant epidemic *K. pneumoniae* clone is XDR, susceptible in most cases only to gentamicin and colistin.\(^3,4\) Besides the KPC-3-encoding pKpQIL plasmid, additional antibiotic resistance genes, such as the *aac(6\(^\prime\))\text{-}Ib* gene conferring resistance to amikacin and the *dhfr* gene conferring resistance to trimethoprim/sulfamethoxazole, were found in all seven isolates belonging to the epidemic strain. These genes were shown to be encoded in a class I integron structure in our strains, as was shown previously in other multidrug-resistant pathogens. Moreover *dhfr* was located on another plasmid, not encoding KPC, carried in these isolates (Figure 1a), as was shown in the case of the 240 kb plasmid present in Kpn557, whereas the *aac(6\(^\prime\))\text{-}Ib* gene was located on the chromosome.

Previous intercontinental genetic comparison between the Israeli epidemic clone and several representative clones from the USA revealed a high degree of similarity between the Israeli clone and a major clone responsible for various outbreaks in New Jersey and in Arizona. The Israeli clone seems to carry a different plasmid from those carried by the US isolates tested belonging to the same clone.\(^3\) Moreover, plasmid pKpQIL appears consistently in all isolates over the years with no apparent change. This plasmid is the largest KPC-3-encoding plasmid reported to date, and we have proved its essential role in carbapenem resistance.

The genetic environment of KPC-2 was extensively analysed in various isolates of carbapenem-resistant *K. pneumoniae* from South America and New York,\(^6\) and recently from KPC-2- and KPC-3-producing strains from New York City.\(^7\) We screened for the presence of the various elements comprising transposon Tn4401 reported previously and confirmed the presence of all of them in our KPC-3-encoding plasmid pKpQIL.

Carbapenem-resistant KPC-producing *K. pneumoniae* is emerging in various parts of the world, and the prevalence of carbapenem-resistant *K. pneumoniae* is increasing in several countries. Various reports document transmission of strains between countries and continents.\(^17,18\) Here we characterized pKpQIL, a KPC-3-encoding plasmid, and its contribution to carbapenem resistance in the Israeli epidemic clone. An international comparison between KPC-encoding plasmids carried by carbapenem-resistant *Klebsiella* strains from different regions worldwide would further assist in the understanding of the spread and evolution of this clinically important antibiotic resistance phenotype. Further studies on plasmid pKpQIL are necessary in order to unravel the reasoning behind the perplexing persistence of this plasmid in the Israeli epidemic *K. pneumoniae* clone.

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**References**

