Outbreak of *Klebsiella pneumoniae* carbapenemase 2-producing *K. pneumoniae* with high *qnr* prevalence in a Chinese hospital

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Forty carbapenem-resistant *Klebsiella pneumoniae* isolates were recovered from 28 patients from various sites in an intensive care unit in Zhejiang Provincial People’s Hospital, China, over a 6 month period. PFGE analysis indicated that the 40 strains were all closely related. The MICs of carbapenems varied from 16 to >256 μg ml⁻¹. Conjugation studies with *Escherichia coli* resulted in the transfer of reduced carbapenem susceptibility from the original isolates. All *K. pneumoniae* and *E. coli* transconjugants produced *K. pneumoniae* carbapenemase 2 (KPC-2), and most of them produced TEM, SHV and CTX-M. Additionally, 27 isolates and 27 *E. coli* transconjugants carried the *qnr* gene (25 were *qnr*B2 and 2 were *qnr*S1). *K. pneumoniae* harboured several plasmids, and *bla*<sub>KPC</sub>-2 was located on a 55 kb plasmid. SDS-PAGE and *ompK35/36* gene sequence analysis of OMPs suggested that porins in *K. pneumoniae* are expressed normally. The MICs of the carbapenems did not change in the presence of CCCP. Thus, production of KPC-2 appears to play an important role in resistance to carbapenems, although other mechanisms may be involved. The *bla*<sub>KPC</sub>-2 gene is associated with several antibiotic-resistance genes, such as *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub> and *qnr*.

**INTRODUCTION**

Extensive use of cephalosporins has led to increased emergence of *Enterobacteriaceae* possessing extended-spectrum β-lactamas. Carbapenems are commonly used to treat serious infections caused by such bacteria. Carbapenem-resistant *Enterobacteriaceae* are uncommon in a clinical setting. However, recently, identification of carbapenem-resistant *Enterobacteriaceae*, especially *Klebsiella pneumoniae* carbapenemase (KPC)-producing bacteria, is increasing. Since the initial report of KPC-1 in *K. pneumoniae* in North Carolina in 2001, this enzyme has spread worldwide. The USA, Israel, South America and China are the areas where KPC-producing bacteria have been isolated most frequently (Nordmann et al., 2009). In China, a *K. pneumoniae* isolate from Hangzhou city producing KPC-2 was first reported in 2007 (Wei et al., 2008). In the same year, we identified KPC-2 in three *Serratia marcescens* isolates from the same city but a different hospital (Zhang et al., 2007). Subsequently, KPC-2 was detected in a *Citrobacter freundii* isolate (Zhang et al., 2008), 21 *S. marcescens*, 10 *K. pneumoniae*, 1 *Escherichia coli* (Cai et al., 2008b) and 1 *Enterobacter cloacae* (Cai et al., 2008a).

In the present report, we describe an outbreak of 40 clinical isolates of carbapenem-resistant *K. pneumoniae* producing KPC-2, TEM, SHV and CTX-M in an intensive care unit (ICU) of another hospital in Hangzhou city, Zhejiang Provincial People’s Hospital. In particular, the prevalence of the *qnr* gene was high (70.0 %) in these KPC-2-producing isolates.

**METHODS**

**Bacterial strains.** Carbapenem-resistant *K. pneumoniae* isolates firstly emerged in June 2007 in a surgery ICU at Zhejiang Provincial People’s Hospital. In the following 7 months, a total of 40 isolates of *K. pneumoniae* with carbapenem resistance were recovered from 28 patients in the same ward. All patients had undergone surgery. All the carbapenem-resistant *K. pneumoniae* isolates during this period were collected for investigation.

Samples from pulmonary infections, wound infections, urinary tract infections and septicemia after surgery were collected for culture. Sputum, urine and blood samples were the most common specimens. Some strains were isolated from the same patient but from a different
clinical sample; these were also included in the investigation. Strains isolated from the same patient during the second hospitalization were also included in the investigation. Species identification was performed with the Vitek system (bioMérieux). Seventy-five per cent of patients were treated with a β-lactam/β-lactamase inhibitor combination (including piperacillin/tazobactam and cefoperazone/sulbactam), and about half of the patients were treated with carbapenems, quinolones or antibiotics with a strong activity against Gram-positive bacteria (including vancomycin, teicoplanin and linezolid) before the organism was isolated (within 2 weeks). Most patients received therapy with a combination of two or three kinds of antibiotics (see Supplementary Table S1 available with the online journal). K. pneumoniae K1 isolated at the 2nd Affiliated Hospital of Zhejiang University, China, and its E. coli transconjugant (Cai et al., 2006b) were used as control strains.

**Antimicrobial-susceptibility testing.** The MICs of 14 antibiotics were determined using the agar dilution method according to Clinical and Laboratory Standards Institute recommendations (CLSI, 2006).

**PFGE analysis.** PFGE typing of K. pneumoniae isolates was performed as described by PulseNet on the website of the Centers for Disease Control and Prevention (http://www.cdc.gov/pulsenet/protocols.htm) in a Rotaphor system 6.0 instrument (Whatman Biometra). The XbaI restriction patterns of the genomic DNA of the isolates were analysed and interpreted according to the criteria described by Tenover et al. (1995).

**Conjugation experiments and analysis of plasmids.** Conjugation experiments were carried out in mixed broth cultures (Cai et al., 2008a). Rifampicin-resistant E. coli EC600 (LacZ+, NalR, RifR) was used as the recipient strain. E. coli transconjugants were selected on Mueller–Hinton agar containing rifampicin (500 μg ml⁻¹) and imipenem (0.3 μg ml⁻¹). The colonies that grew on the selecting medium were picked up and identified using the Vitek system. Plasmids from K. pneumoniae isolates and E. coli transconjugants were extracted using an AxyPrep plasmid miniprep kit (Axygen Scientific) and examined by electrophoresis.

**IEF of β-lactamase.** The crude β-lactamase extracts of K. pneumoniae isolates and their E. coli transconjugants were prepared by ultrasonic treatment of bacterial cells. IEF was carried out on a PhastGel polyacrylamide gel (pH 3–9; Amersham Biosciences) using the PhastSystem (Pharmacia Biotech) following the method of Mathew et al. (1975). β-Lactamase activity was visualized by staining the gel with (500 μg ml⁻¹) nitrocefin (Oxoid). The isoelectric point (pI) was determined after comparison with those of known β-lactamases: TEM-28 (pI 6.1), SHV-7 (pI 7.6) and ACT-1 (pI 9.0).

**PCR amplification and DNA sequence analysis of drug-resistance genes.** Plasmid DNA or genomic DNA from K. pneumoniae isolates and E. coli transconjugants was used as the template depending on the type of target gene in the PCR amplification. The primers used to amplify blapEM, blapSHV and blapCTX-M (Yu et al., 2007), plasmid-mediated AmpC genes (Pérez-Pérez & Hanson, 2002), plasmid-mediated quinolone-resistance genes (qnrA, qnrB and qnrS (Robischek et al., 2006) and aac(6’)-Ib-cr (jiang et al., 2008)), blapKPC (Yigit et al., 2001), other class A carbapenemases, blapNMC, blapM, blapAM and blapGES, metallo-β-lactamase, blapIMP-1, blapIMP-2, blapVIM-1 and blapVIM-2, and a class D carbapenemase blapOXA-48 (Quenan & Bush, 2007) were used as PCR primers. The reaction was conducted in a T-personal thermal cycler (Whatman Biometra). PCR amplification products were purified and sequenced directly using an ABI3730 Sequence (Applied Biosystems). DNA sequences were compared with the reported nucleotide sequences from GenBank using the BLASTN program (http://blast.ncbi.nlm.nih.gov).

**Analysis of outer-membrane proteins (OMPs).** The OMPs of K. pneumoniae strains KP1, KP6, KP11, KP29, KP39 and K. pneumoniae ATCC 13883 were isolated as described by Hernández-Alles et al. (1999). OMP profiles were determined by SDS-PAGE using 11.6% acrylamide/0.4% bisacrylamide/0.1% SDS in the running gel. Samples were boiled for 5 min in sample buffer before electrophoresis. The 0.75 mm thick mini gel was run at a constant current of 20 mA in a Mini Protein 3 slab electrophoresis cell (Bio-Rad). Proteins were visualized by staining with Coomassie brilliant blue (2.5 μg ml⁻¹).

The ompK35 and ompK36 genes of strains KP1, KP6, KP11, KP29 and KP39 were amplified by PCR (Kaczmarek et al., 2006). The products were sequenced and the sequences compared with reported sequences from GenBank database using BLASTN.

**Efflux mechanism.** The efflux pump activity was examined by inhibition assays with carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma). The MICs of carbapenems were determined by dilution on Mueller–Hinton agar, with and without CCCP (final concentration 50 μM) (Hasdemir et al., 2004).

**RESULTS AND DISCUSSION**

**Antimicrobial susceptibility**

Forty K. pneumoniae isolates showed various levels of resistance to carbapenems. The MICs for imipenem, meropenem and ertapenem for these 40 isolates were 16 to 128, 32 to >256 and 64 to >256 μg ml⁻¹, and were much higher than those of K. pneumoniae K1 (see Supplementary Table S2 available with the online journal). All of the K. pneumoniae isolates had high-level resistance to penicillins, cephalosporins, cefotixin, aztreonam and quinolones, and were susceptible to aminoglycosides (except for KP25, KP33 and KP39). Interestingly, the MICs of ceftazidime were much more varied: for the majority of isolates, the MIC values were >256 μg ml⁻¹, while for a portion of them the MIC values were 32 μg ml⁻¹.

**PFGE typing**

As shown in Fig. 1, the 40 K. pneumoniae isolates had similar PFGE patterns. No more than five band differences were observed among the subtypes, suggesting that all isolates were closely related or possibly related to each other, and demonstrating the possibility of intra-hospital clonal dissemination of carbapenem-resistant K. pneumoniae. The PFGE patterns of 2 KPC-2-producing K. pneumoniae isolated from the 2nd Affiliated Hospital of Zhejiang University, C1 and C2, were quite different from those of the 40 K. pneumoniae isolates from Zhejiang Provincial People’s Hospital, indicating that they were genetically unrelated.

**Transfer of carbapenem resistance and plasmid analysis**

Carbapenem resistance was transferred from K. pneumoniae to E. coli EC600 after conjugation. All E. coli transconjugants exhibited significantly reduced carbapenem

978

Journal of Medical Microbiology 60

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susceptibility. The MICs of imipenem, meropenem and ertapenem ranged from $\leq 0.125$ to 1–4, from 0.5 to 4 and from 1 to 8 $\mu$g ml$^{-1}$, respectively. The antimicrobial-susceptibility patterns of E. coli transconjugants were similar to those of the donor clinical isolates of K. pneumoniae (see Supplementary Table S2 available with the online journal). They were resistant to penicillins and aztreonam, and were resistant or intermediately resistant to cefoxitin, but were susceptible to quinolones (except for the E. coli transconjugants of KP11 and KP40) and aminoglycosides. For the cephalosporins, however, the E. coli transconjugants showed various levels of resistance. The MICs of ceftazidime for 12 E. coli transconjugants were much higher than those of the other E. coli transconjugants, whilst the MICs of cefotaxime and cefepime for some E. coli transconjugants were lower than those of other transconjugants.

As shown in Fig. 2, the K. pneumoniae isolates had similar plasmid profiles. Most isolates showed five bands following electrophoresis, with sizes of approximately 55, 5.6, 4.2, 3.9 and 3.2 kb, although a few isolates lacked the 3.9 kb band. All E. coli transconjugants acquired a plasmid of ~55 kb, and some had an additional band with a size of approximately 4.2 or 3.9 kb.

**IEF analysis**

The IEF results showed that the recipient strain E. coli EC600 did not produce any $\beta$-lactamase, and had no pl band (data not shown). All K. pneumoniae isolates had a common band with $\beta$-lactamase activity at a pl of ~6.7. The majority had extra bands with pls of 5.4 and 7.9, and a band with a pl of 7.3 was observed in some isolates. The pl 6.7 band was found uniformly in all E. coli transconjugants. To our surprise, many E. coli transconjugants also produced bands of pl 5.4, and some of them produced both 5.4 and 7.9 bands, but few had the pl 7.3 band (Fig. 3).

**PCR and DNA sequence analysis**

All K. pneumoniae isolates and E. coli transconjugants produced KPC-2, and most produced additional $\beta$-lactamases such as TEM-1, SHV-11/-12 and CTX-M-14/-3. A total of 39 isolates and 38 E. coli transconjugants produced TEM-1, 40 isolates produced SHV (11 were SHV-11, 29 were SHV-12, and 12 E. coli transconjugants were positive for SHV-12), 38 isolates produced CTX-M (37 were CTX-M-14, 1 was CTX-M-3, and 32 E. coli transconjugants were positive for CTX-M-14), and 27 isolates carried the qnr gene (25 were qnrB2, 2 were qnrS1, and 27 E. coli transconjugants all positive for qnr) (see Supplementary Table S1 available with the online journal). SHV-1 is chromosomally encoded in the majority of K. pneumoniae isolates. SHV-11 is a derivative of SHV-1 and is a non-extended-spectrum $\beta$-lactamase. In this study, strains carrying the plasmid-mediated SHV-12 should also be positive for the chromosomally mediated SHV-11. No other carbapenemase tested in this experiment was detected besides KPC. $\beta$-Lactamases with pls of 6.7, 5.4 and 7.9 were consistent with KPC, TEM and CTX-M, respectively. The pl 7.3 band seemed to be inconsistent with SHV-11 and SHV-12 (usually pl 7.6 and 8.2), and the reason for this remains unclear. The level of resistance to ceftazidime for E. coli transconjugants was related to the genotype of SHV, as is clear from the PCR results for resistance genes and MIC values (Supplementary Tables S1 and S2 available with the online journal). Other mechanisms may contribute towards carbapenem resistance.

The MICs of the carbapenems for the K. pneumoniae isolates were significantly higher than those of the E. coli transconjugants and K. pneumoniae K1. We presumed that other carbapenem-resistance mechanisms might be involved. Therefore, analysis of outer-membrane permeability and the efflux pump activity was performed. The SDS-PAGE
results revealed that five *K. pneumoniae* isolates did not lack OMPs. These five isolates and *K. pneumoniae* ATCC 13883 expressed four major OMPs, with molecular masses of approximately 49, 40, 36 and 32 kDa, which corresponded to LamB, OmpK36, OmpK35 and OmpA, respectively (Fig. 4). Amplification and sequencing of the *ompK35/K36* genes indicated that the five isolates had identical gene sequences. There were a few silent point mutations in the *ompK35* gene compared with that of *K. pneumoniae* KT755 (GenBank accession no. AJ011501), and there were several point mutations and three small DNA fragment insertions (6, 6 and 21 bp) in the *ompK36* gene compared with that of *K. pneumoniae* C3 (GenBank accession no. Z33506). However, the ORFs of both *ompK35* and *ompK36* genes were normal. The MICs of imipenem, meropenem and ertapenem with CCCP for *K. pneumoniae* were identical to those without CCCP, indicating that carbapenem efflux was apparently not involved in these *K. pneumoniae* isolates. Other mechanisms involved with the high level of resistance to carbapenems remained unclear.

**High prevalence of the qnr gene in KPC-2-producing *K. pneumoniae***

KPCs are currently the most frequent class A carbapenemase and have become a matter of great concern. These enzymes have been detected in many genera and species of *Enterobacteriaceae* and *Pseudomonas* spp. in many countries (Nordmann et al., 2009). In Hangzhou city, a total of 38 KPC-2-producing bacteria were isolated from two hospitals at Zhejiang University (Cai et al., 2008a, b; Wei et al., 2007; Zhang et al., 2007, 2008). Recently, within 7 months, we isolated 40 KPC-2-producing *K. pneumoniae* in an ICU of another hospital in Hangzhou. These isolates showed high-level resistance to carbapenems and other β-lactams. Further studies demonstrated several resistance genes, such as TEM, SHV and CTX-M, and especially the *qnr* gene, in these *K. pneumoniae* isolates. Worryingly, many of the *E. coli* transconjugants were positive for more than one resistance gene beside KPC-2, suggesting the coexistence of multiple resistance genes on the same plasmid, and the plasmids were able to self-transfer to other bacteria, shown at least for *E. coli*, which might cause the wide spread of multiple drug resistance. In previous studies, all *bla*KPC-encoded plasmids found in Hangzhou carried only the *bla*KPC-2 gene (Cai et al., 2008a, b; Wei et al., 2007; Zhang et al., 2007, 2008). Many *bla*KPC-encoded plasmids also encode *bla*TEM-1 (Cuzon et al., 2008; Dortet et al., 2008; Leavitt et al., 2007; Miriagou et al., 2003; Naas et al., 2005, 2008), some encode *bla*SHV (Robicsek et al., 2006; Yigit et al., 2003), *bla*CTX-M (Cuzon et al., 2008; Dortet et al., 2008; Leavitt et al.,

![Fig. 2. Plasmid profiles of (a) partial *K. pneumoniae* (KP) and (b) their *E. coli* transconjugants (trans). Plasmids from *E. coli* V517 were used as molecular mass references. N, negative control (*E. coli* EC600).](image-url)

![Fig. 3. IEF patterns of crude β-lactamase extracts from partial *K. pneumoniae* and their corresponding *E. coli* transconjugants (t). P, *K. pneumoniae* K1 isolated from the 2nd Affiliated Hospital of Zhejiang University (producing KPC-2, TEM-1, SHV-11 and CTX-M-14); t, *E. coli* transconjugant; M, strain producing TEM-28 (pl 6.1), SHV-7 (pl 7.6) and ACT-1 (pl 9.0).](image-url)
2007; Monteiro et al., 2009; Naas et al., 2008; Tsakris et al., 2008; Yigit et al., 2003) and some encoded qnr (Chmelnitsky et al., 2008; Endimiani et al., 2008; Mendes et al., 2008). Unlike these, most blaKPC-encoding plasmids in this study were associated with four drug-resistance genes. Notably, ten of them were associated with five resistance genes, encoding KPC-2, TEM-1, SHV-12, CTX-M-14 and QnrB2. Moreover, we identified qnrS associated with blaKPC on the same plasmid in K. pneumoniae for what is believed to be the first time. These findings warn us that novel combinations of transferable resistance determinants have emerged and could result in a serious problem regarding therapy and control. The qnrS1-positive E. coli transconjugants were immediately resistant to ciprofloxacin, whereas the qnrB2-positive transconjugants were susceptible. Qnr determinants alone may not confer resistance to quinolones, but they can supplement other quinolone-resistance mechanisms (Martínez-Martínez et al., 2003; Poirel et al., 2006).

KPCs alone confer reduced susceptibility to carbapenems but are not sufficient to achieve full resistance, and other mechanisms, e.g. porin loss, are usually required (Nordmann et al., 2009). The K. pneumoniae isolates in this study showed various levels of resistance to carbapenems, and the MICs of two isolates (KP6 and KP29) were >128 μg ml⁻¹. However, the rare mechanism of carbapenem resistance in Enterobacteriaceae, the efflux pump and the common mechanism of porin deficiency were not involved. This suggests that other unknown mechanisms may contribute towards carbapenem resistance in these K. pneumoniae isolates, which requires further study.

REFERENCES


Fig. 4. SDS-PAGE analysis of OMPs from partial K. pneumoniae isolates and K. pneumoniae ATCC 13883. M, protein molecular mass standard (MBI Fermentas).


