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 *qnrB2* and 2 were *qnrS1*). *K. pneumoniae* harboured several plasmids, and *bla*_{KPC-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*_{KPC-2} gene is associated with several antibiotic-resistance genes, such as *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M} and *qnr*.

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INTRODUCTION

Extensive use of cephalosporins has led to increased emergence of *Enterobacteriaceae* possessing extended-spectrum β-lactamases. 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.*, 2007). 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 septicaemia 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

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ICU, intensive care unit; KPC, *Klebsiella pneumoniae* carbapenemase; OMP, outer-membrane protein; pI, isoelectric point.

Tables with the clinical details and details of the resistance genes of the isolates and the antibiotic susceptibility results are available as supplementary data with the online version of this paper.

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.*, 2008b) 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 *Xba*I 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⁻, Nal^R, Rif^R) was used as the recipient strain. *E. coli* transconjugants were selected on Mueller–Hinton agar containing rifampicin (500 μ g ml⁻¹) and imipenem (0.3 μ g 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 μ g 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 *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} (Yu *et al.*, 2007), plasmid-mediated AmpC genes (Pérez-Pérez & Hanson, 2002), plasmid-mediated quinolone-resistance genes [*qnrA*, *qnrB* and *qnrS* (Robicsek *et al.*, 2006) and *aac(6')*-Ib-cr (Jiang *et al.*, 2008)], *bla*_{KPC} (Yigit *et al.*, 2001), other class A carbapenemases, *bla*_{NMC}, *bla*_{SME}, *bla*_{IMI} and *bla*_{GES}, metallo- β -lactamase, *bla*_{IMP-1}, *bla*_{IMP-2}, *bla*_{VIM-1} and *bla*_{VIM-2}, and a class D carbapenemase *bla*_{OXA-48} (Queenan & Bush, 2007) were as described. The reaction was conducted in a T-personal thermal cycler (Whatman Biometra). PCR amplification products were purified and sequenced directly using an ABI3730 Sequencer (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-Allés *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 μ g 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 μ M) (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 μ g 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, cefoxitin, 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 μ g ml⁻¹, while for a portion of them the MIC values were 32 μ g 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

Fig. 1. PFGE patterns of chromosomal DNA restriction fragments from *K. pneumoniae* isolates. Lanes 1–40, carbapenem-resistant *K. pneumoniae* KP1 to KP40, respectively; C1 and C2, two KPC-2-producing *K. pneumoniae* isolated from the 2nd Affiliated Hospital of Zhejiang University.

susceptibility. The MICs of imipenem, meropenem and ertapenem ranged from ≤ 0.125 to 1–4, from 0.5 to 4 and from 1 to 8 $\mu\text{g ml}^{-1}$, 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 ceftazidime, 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 β -lactamase, and had no pI band (data not shown). All *K. pneumoniae* isolates had a common band with β -lactamase activity at a pI of ~6.7. The majority had extra bands with pIs of 5.4 and 7.9, and a band with a pI of 7.3 was observed in some isolates. The pI 6.7 band was found uniformly in all *E. coli* transconjugants. To our surprise, many *E. coli* transconjugants also produced bands of pI 5.4, and some of them produced both 5.4 and 7.9 bands, but few had the pI 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 β -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 were 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 β -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. β -Lactamases with pIs of 6.7, 5.4 and 7.9 were consistent with KPC, TEM and CTX-M, respectively. The pI 7.3 band seemed to be inconsistent with SHV-11 and SHV-12 (usually pI 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

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 co-existence 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}-encoding 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}-encoding 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.*,

980 *Journal of Medical Microbiology* 60

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

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 *bla*_{KPC}-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 *bla*_{KPC} 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 *qnrS*-positive *E. coli* transconjugants were intermediately 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 $>128 \mu\text{g ml}^{-1}$. 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.

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