Nosocomial spread of multi-resistant *Klebsiella pneumoniae* containing a plasmid encoding multiple β-lactamases

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Six *Klebsiella pneumoniae* isolates that exhibited resistance to a wide spectrum of antibiotics were recovered from the intensive care units in the First Affiliated Hospital, Zhejiang University, Hangzhou, China. All isolates contained two plasmids of approximately 95 kb and 200 kb. The 95 kb plasmid was shown to be transferable by conjugation experiments. Isoelectric focusing patterns of the β-lactamases extracted from the six transconjugants were identical, displaying five pl bands: 5.4, 7-75, 8.0, 8.2 and 8.4. The band corresponding to a pI of 7-75 could be inhibited by cloxacillin but not clavulanic acid, while the other bands could be inhibited by clavulanic acid but not cloxacillin. The 95 kb plasmid was digested with HindIII and a recombinant plasmid pT94B was obtained. The insert was found to contain *bla*<sub>DHA-1</sub>, regulatory gene *ampR* and an insertion element (IS26), which was downstream of *bla*<sub>DHA-1</sub>. PCR and DNA sequencing results confirmed that the 95 kb plasmid encoded at least four β-lactamase genes: *bla*<sub>TEM-1</sub>, *bla*<sub>SHV-12</sub>, *bla*<sub>CTX-M-3</sub> and *bla*<sub>DHA-1</sub>. Epidemiological typing by PFGE of the six clinical isolates of *K. pneumoniae* demonstrated identical genotypic patterns. In conclusion, all results indicated that the six multi-drug resistant clinical isolates of *K. pneumoniae* most probably originated from one clone and caused a localized epidemic in the intensive care units.

INTRODUCTION

Many β-lactamase genes have been found in *K. pneumoniae* plasmids, including those encoding extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases, inhibitor-resistant TEM β-lactamases and metalloenzymes. These enzymes confer resistance to various antimicrobial agents including the third and fourth generation cephalosporins, cephemycins, monobactam β-lactamase, β-lactamase/inhibitor combinations and carbapenems (Essack *et al.*, 2004; Hanson *et al.*, 1999; Lemozy *et al.*, 1995; Moland *et al.*, 2003; Poirel *et al.*, 2004; Yan *et al.*, 2001). Most importantly, the number of resistance genes carried on the plasmids of multi-resistant *K. pneumoniae* is usually more than one, and occasionally as many as five genes are reported (Essack *et al.*, 2004; Hanson *et al.*, 1999; Poirel *et al.*, 2004; Yan *et al.*, 2001). In fact, coexistence of broad-spectrum β-lactamases with ESBLs, ESBLs with AmpC β-lactamase, multiple ESBLs or ESBLs with metallo-β-lactamase has become common in multi-resistant *K. pneumoniae* (Essack *et al.*, 2004; Hanson *et al.*, 1999; Yan *et al.*, 2001). Of these enzymes, ESBLs were the most prevalent in *K. pneumoniae*, frequently encoded on large plasmids with sizes of 80–160 kb.

DHA-1 is an AmpC-type β-lactamase that shares 98.7 % amino acid similarity with the chromosomal AmpC enzyme from *Morganella morganii* and was originally described by Verdet *et al.* (2000) on a complex **sll**-type integron. This integron was responsible for the transfer of *bla*<sub>DHA-1</sub> and the corresponding *ampR* gene from the chromosome of *M. morganii* to *Salmonella enteritidis* (Verdet *et al.*, 2000). In this study, we report the finding and characterization of a plasmid containing multiple resistance genes, including one encoding the plasmid-mediated AmpC β-lactamase (DHA-1), and the downstream IS26 element, as well as those encoding a broad-spectrum β-lactamase and two ESBLs.

METHODS

**Bacterial strains.** Six multi-resistant *K. pneumoniae* isolates were obtained from five patients between May and August 2000 in the intensive care unit of Zhejiang University’s First Affiliated Hospital. The isolates were identified by the API 20E system. The MICs of ceftazidime with or without clavulanic acid for these six isolates were all above 256 μg ml<sup>−1</sup>. Streptomycin-resistant *Escherichia coli* C600 was used as the recipient in conjugation experiments. *E. coli* DH5α and plasmid pGEM-T Easy (Promega) were used in cloning experiments as the host strain and vector, respectively.
Susceptibility testing. The MICs of antimicrobial agents for clinical isolates of *K. pneumoniae* and transconjugants were determined by E-test (AB Biodisk) according to the manufacturer’s instructions. The antimicrobial agents tested were imipenem, ceftazidime, ceftriaxone/clavulanic acid, cefotaxime, cefoxazime, ceftriaxone, cefepime, cefoxitin, ticarcillin/clavulanic acid, piperacillin/tazobactam, cefoperazone/sulbactam, ciprofloxacin, gentamicin and amikacin. *E. coli ATCC 25922 and E. coli ATCC 35218 were used as quality control strains.

**PFGE.** Genomic DNA was analysed by PFGE after digestion with *XbaI* (Sangon), using the contour-clamped homogeneous electric field (CHEF) technique (Goughy et al., 1994). DNA fragments were separated by electrophoresis in 1% agarose III (Sangon) in 0.5 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) with CHEF apparatus (CHEF MAPPER XA; Bio-Rad) at 14°C and 6 V cm⁻¹ and with alternating pulses at a 120° angle in a 2–40 s pulse time gradient for 22.5 h. DNA from λ DNA-PFGE markers (Amersham) was used as size markers. Restriction patterns were interpreted by the criteria proposed by Tenover et al. (1995).

Transconjugation, plasmid extraction and plasmid fingerprints. Conjugation was carried out by a broth method as described elsewhere (Yan et al., 2001). The plasmids of clinical isolates and transconjugants were extracted by the method of Kado and Liu (1981), with plasmids 40R646 (58 kb), 28R823 (221 kb), 40R448 (61 kb), 40R268 (98 kb) and RT641 (91 kb) as plasmid size markers (Ling et al., 2000). Plasmid DNA of the six transconjugants was extracted with Qiagen Plasmid Maxi Kit (Qiagen) and digested with EcoRI and EcoO109I.

**Isoelectric focusing.** Isoelectric focusing was performed according to a published protocol (Coudron et al., 2000). Isoelectric focusing was performed using the PhastSystem (Pharmacia) in an ampholine gel (pH 3.0–9.0; Pharmacia), and SHV-18 (pl 7.8), TEM-12 (pl 5.25), SHV-1 (pl 7.61) and ACT-1 (pl 9.0) as pl markers. Filter paper containing either 0.5 mM clavulanic acid (Jiangsu Simcere) or 0.5 mM cloxacillin (Sigma) was used as an inhibitor for 30 s in isoelectric focusing test before staining with nitrocefin.

**PCR amplification and sequencing.** Isolates of clinical *K. pneumoniae* and transconjugants were amplified by the standard PCR (Sambrook et al., 1989). Oligonucleotide primers for the TEM gene were designed according to the nucleotide sequence of TEM-1 (GenBank accession no. AF188200): TEM-3, 5'-TTAGACGTCAGGTGGCACTT-3' (nucleotides 991–1010) and SHV-1 (pl 7.8), TEM-12 (pl 5.25), SHV-1 (pl 7.61) and ACT-1 (pl 9.0) as pl markers. Filter paper containing 0.5 mM clavulanic acid (Jiangsu Simcere) or 0.5 mM cloxacillin (Sigma) was used as an inhibitor for 30 s in isoelectric focusing test before staining with nitrocefin.

**Cloning and sequencing of the plasmid-mediated AmpC gene.** The plasmid of transconjugant KP6 was extracted by Qiagen Plasmid Maxi Kit and digested with *HindIII*. DNA fragments containing the AmpC gene were cloned into the pGEM-T vector. Recombinants were selected on MacConkey agar containing 10 μg cefoxitin ml⁻¹. The entire insert fragment from the recombinant plasmid was completely sequenced by the walking method with an automated sequencer.

**Nucleotide sequence accession numbers.** The nucleotide sequences described in this study can be found in GenBank under accession no. AJ705809, a 6432 bp sequence containing DHA-1.

**RESULTS AND DISCUSSION**

The six clinical isolates of *K. pneumoniae* all had a similar antibiotic-resistance pattern; they were resistant to cefazidine, cefotaxime, ceftriaxone, gentamicin, amikacin, ciprofloxacin, aztreonam, cefoxitin and ticarcillin/clavulanic acid, and intermediate resistant to piperacillin/tazobactam, cefoperazone/sulbactam and cefepime. Among the test drugs, the isolates were susceptible to imipenem only.

Conjugation of each of the six *K. pneumoniae* isolates with *E. coli C600* was successful, with a transfer frequency of 10⁻⁶–10⁻⁷. The plasmid patterns of the six clinical isolates were all identical, exhibiting two plasmids of approximately 95 kb and 200 kb (Fig. 1). Only the plasmid of approximately 95 kb was extracted from each of the six transconjugants. DNA fingerprints of plasmids from the six transconjugants digested with either EcoRI or EcoO109I were identical (Fig. 2). The drug-susceptibility profiles of the transconjugants were similar to the clinical isolates, except for the susceptibility to amikacin and ciprofloxacin.

PCR and nucleotide sequence analysis found that the six clinical isolates of *K. pneumoniae* and their corresponding transconjugants with their corresponding transconjugants. Ma, 119 kb, 91 kb and 58 kb markers; Mb, 98 kb marker. Lanes 1, 3, 5, 7, 9 and 11, plasmids of transconjugants from clinical isolates of *K. pneumoniae* PK1, PK2, PK3, PK4, PK5 and PK6, respectively; lanes 2, 4, 6, 8, 10 and 12, plasmids from clinical isolates of *K. pneumoniae* PK1, PK2, PK3, PK4, PK5 and PK6, respectively.

**Fig. 1.** Plasmid profiles of six clinical isolates of *K. pneumoniae* and their corresponding transconjugants. Ma, 119 kb, 91 kb and 58 kb markers; Mb, 98 kb marker. Lanes 1, 3, 5, 7, 9 and 11, plasmids of transconjugants from clinical isolates of *K. pneumoniae* PK1, PK2, PK3, PK4, PK5 and PK6, respectively; lanes 2, 4, 6, 8, 10 and 12, plasmids from clinical isolates of *K. pneumoniae* PK1, PK2, PK3, PK4, PK5 and PK6, respectively.

**Fig. 2.** Agarose gel electrophoresis analysis of plasmids of six transconjugants digested with EcoRI and EcoO109I. Ma, λ-HindIII digest DNA Marker; Mb, DNA Marker DL15000. Lanes 1–6, restriction patterns of EcoRI; lanes 7–12, restriction patterns of EcoO109I; lanes 1 and 7, pPK1; lanes 2 and 8, pPK2; lanes 3 and 9, pPK3; lanes 4 and 10, pPK4; lanes 5 and 11, pPK5; lanes 6 and 12, pPK6. (The plasmids extracted from the six transconjugants were designated pPK1 to pPK6.)
Plasmid-encoded multiple β-lactamases in K. pneumoniae

transconjugants carried \( \text{bla}_{\text{TEM-1}}, \) \( \text{bla}_{\text{SHV-12}}, \) \( \text{bla}_{\text{CTX-M-3}} \) and \( \text{bla}_{\text{DHA-1}} \). Isoelectric focusing patterns of the β-lactamases extracted from the transconjugants were identical to the donor strains, displaying five pI bands: 5.4, 7.75, 8.0, 8.2 and 8.4. The band corresponding to a pI of 7.75 could be inhibited by cefoxitin but not clavulanic acid, while the other bands could be inhibited by clavulanic acid but not cefoxitin. Thus, the pI 7.75 band probably represented the DHA-1 β-lactamase. The pI bands of 5.4, 8.2 and 8.4 accounted for the TEM-1, SHV-12 and CTX-M-3 enzymes, respectively. The band of pI 8.0 was relatively weak. To confirm its genotype, a set of primers was used to detect the β-lactamase genes of OXA-15, OXA-23, CTX-M-2, CTX-M-8, CTX-M-9, TOHO-2 and VEB-1, but the PCR results were negative.

Since the first report of SHV-2 ESBLs in China in 1994, the frequency of ESBLs has been on the rise in this country (Cheng & Chen, 1994). In particular, ESBL-producing bacteria have become one of the most difficult clinical problems in recent years. CTX-M β-lactamases are the predominant ESBLs in China, including CTX-M-3, CTX-M-9, CTX-M-13, CTX-M-14 and CTX-M-18 (Chanawong et al., 2003; Ji et al., 2004; Li et al., 2003; Wang et al., 2003). Moreover, some new CTX-M β-lactamases have also been reported in China in recent years, including CTX-M-22 (AY080894), CTX-M-24 (AY143430), CTX-M-29 (AY267213) and CTX-M-32 (AY421962), which are included in GenBank (accession nos in parentheses). The main reason for the prevalence of CTX-M β-lactamases in China may be the widespread use of certain third generation cephalosporins such as cefotaxime and ceftriaxone, whilst ceftazidime has been less frequently prescribed. Antibiotic selection pressure probably contributes to the increasing prevalence of cefotaxime- and ceftriaxone-hydrolysing CTX-M β-lactamase in clinical settings. Another consideration may be the prevalence of CTX-M β-lactamases, including TOHO-1 (Yagi et al., 2000), CTX-M-2, CTX-M-3 (Yamasaki et al., 2003) and CTX-M-14 (Paï et al., 2001), in neighbouring countries such as Japan and Korea, from where they may spread to China through international travel.

In the study, a recombinant plasmid pT948 containing a plasmid-mediated AmpC β-lactamase gene was obtained from the 95 kb plasmid of a transconjugant. A 6432 bp DNA sequence was obtained by nucleotide sequencing. This fragment contained six ORFs, IS26, orf-2, \( \text{bla}_{\text{DHA-1}} \), \( \text{ampR} \), \( \text{qacEA1} \) and \( \text{sull} \) (Fig. 3), which exhibited a high level of identity with pPON-1 of \( M. \) morganii (Poirel et al., 1999) and pSAL-1 of \( S. \) enteritidis (Verdet et al., 2000) (Fig. 3). The orf-2 in the inserted fragment in this study exhibited 98 % identity with the orf-1 in \( M. \) morganii, which is a conserved sequence in \( M. \) morganii species with an unknown function. Fortineau et al. (2001) suggested that the reason why DHA-1 could be induced might be due to the large-size DNA fragment excised from \( M. \) morganii, involving not only \( \text{ampR} \) but also \( \text{ampC} \). The presence of the orf-2 gene in this study indicated further that the plasmid-mediated DHA-1 may originate from the \( M. \) morganii chromosome. The \( \text{qacEA1} \) and \( \text{sull} \) genes were found in the upstream region of the \( \text{bla}_{\text{DHA-1}} \) genes. Frequently, these genes were found in the typical 3′-conserved sequence (3′-CS) of integrin I. However, we were not able to identify the 5′-conserved sequence (5′-CS) in the upstream region by PCR. IS26 was found downstream of the \( \text{bla}_{\text{DHA-1}} \) gene; this is commonly related to the transmission of β-lactamase genes such as CFE-1, ACC-1 and SHV-2a (Kim et al., 2002; Nadjar et al., 2000; Nakano et al., 2004).

The genotype and phenotype analysis suggested that the six prevalent \( K. \) pneumoniae isolates in the hospital originated from one clone. PFGE yielded identical profiles, consisting of 18 bands, when chromosomal DNA of each of the test isolates was digested with \( XbaI \). Each of the six isolates had the same plasmid profile of two plasmids with sizes of 95 kb and 200 kb (Fig. 1). The plasmid profiles of their corresponding transconjugants were also identical. Furthermore, these isolates had similar antibiotic-resistance patterns.

Nucleotide sequence analysis of PCR products confirmed that the 95 kb plasmid in the transconjugants harboured at least four β-lactamase genes including \( \text{bla}_{\text{TEM-1}}, \) \( \text{bla}_{\text{SHV-12}}, \) \( \text{bla}_{\text{CTX-M-3}} \) and \( \text{bla}_{\text{DHA-1}} \). Multi-resistant \( K. \) pneumoniae containing plasmids encoding multi-drug-resistance genes have been reported worldwide. The prevalence of such multi-resistant strains in intensive care units is a serious event even though these strains were not found in subsequent surveillance. Carbapenems were the antibiotic of choice for managing these bacteria. Unfortunately, a plasmid-mediated β-lactamase conferring imipenem resistance has also been reported on the plasmid of \( K. \) pneumoniae (Moland et al., 2003; Poirel et al., 2004; Yan et al., 2001). These events must make clinicians highly aware of the problem of antibiotic resistance in \( K. \) pneumoniae.

![Fig. 3. Comparison of the structural genes of (A) pPON-1 (\( M. \) morganii), (B) DHA-1 (this study) and (C) pSAL-1 (\( S. \) enteritidis).](http://jmm.sgmjournals.org)
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