Molecular Characterization of Extended-Spectrum β-Lactamases Produced by Clinical Isolates of Enterobacter cloacae from a Teaching Hospital in China

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SUMMARY: Of 59 clinical isolates of Enterobacter cloacae from a teaching hospital in Sichuan, China, 18 isolates were shown to be resistant to oximino cephalosporins and aztreonam. Enterobacterial repetitive consensus PCR revealed that these isolates comprised 7 distinct genotypes. The presence of plasmids in the 18 clinical isolates was revealed by conjugational transfer of plasmids from E. cloacae to Escherichia coli with the further isolation of the plasmids in the transconjugants. Subsequent nucleotide sequencing and β-lactamase isoelectric focusing indicated that the plasmids encoded blaSHV, blaCTX-M, and/or blaTEM genes, including genes for CTX-M-22 (13 strains), TEM-1 (12 strains), TEM-29 (1 strain), TEM-141 (1 strain), TEM-157 (1 strain), SHV-5 (1 strain), SHV-12 (1 strain), and SHV-70 (1 strain). The widespread presence of extended-spectrum β-lactamases in E. cloacae isolated from the southwest of China was likely due to the dissemination of resistance plasmids with the predominant genotype of blaCTX-M-22.

INTRODUCTION
Resistance to extended-spectrum β-lactam antibiotics in Gram-negative bacteria has been emerging rapidly worldwide over the last two decades and has been attributed predominantly to the production of β-lactamases, including plasmid-encoded extended-spectrum β-lactamases (ESBLs) (1-3). Indeed, more than 200 different ESBLs have been described to date and these enzymes have been found worldwide in many bacteria, particularly in the family of Enterobacteriaceae (2).

In China, extended spectrum β-lactams such as the oximino cephalosporins (e.g., ceftazidime and cefotaxime) are widely prescribed, and thus ESBL-mediated β-lactam resistance has increasingly been reported in recent years (4-9). However, the majority of the studies in China have involved phenotypic identifications of the ESBL-producing isolates of Escherichia coli and Klebsiella pneumoniae (4-7), while the molecular basis of β-lactam resistance in other Gram-negative bacteria has been less clear. In recent studies (8,10), we have reported a high prevalence of ESBL-producing isolates of Enterobacter cloacae as well as the enzymatic characterization of the plasmid-encoded ESBLs, SHV-70 and CTX-M-22. To understand the widespread occurrence of the ESBL phenotype in E. cloacae isolates derived from north Sichuan, we examined the genetic similarity between these ESBL producers and characterized the ESBL enzyme families produced by these strains.

MATERIALS AND METHODS
Bacterial strains and plasmids: During the period of May 2003 to September 2004, 59 isolates of E. cloacae were collected from the patients with bacteremia, upper/lower respiratory tract infections, urinary tract infections or surgical wounds in the intensive care units at the First Teaching Hospital of the North Sichuan Medical College, a hospital with more than 1,000 beds. These isolates were identified using an API20-E system (Bio-Merieux, Marcy l’Etoile, France). The strains resistant to ceftazidime and/or cefotaxime were further studied for resistance to β-lactams, and E. coli JM109 was used as the host in conjugation.

Antibiotics and susceptibility testing: The antibiotics used in this study were obtained from their respective manufacturers as follows: ampicillin and piperacillin from Roche, Shanghai, China; cefazolin, cefoperazone, cefotaxime, ceftazidime, and sulbactam from Hayao Co., Haerbin, China; tazobactam from Gloria Technology Co., Ltd., Haerbin, China; cefamandole from Asia Pioneer, Shanghai, China; imipenem from Merck Sharp & Dohme Ltd., Hong Kong, China; cefepime and aztreonam from Bristol-Myers Squibb Co., Shanghai, China; and nitrocefin from BBL, Cockeysville, Md., USA. Antibiotic susceptibility was determined by serial twofold dilution in Mueller-Hinton II agar with an inoculum of 10⁴ CFU/spot according to the Clinical and Laboratory Standards Institute (11). E. coli ATCC25922 and Pseudomonas aeruginosa ATCC27853 were used as ESBL-negative controls, and K. pneumoniae ATCC700603 was included as an ESBL-positive control.

Enterobacterial repetitive intergenic consensus (ERIC)-PCR:ERIC-PCR was performed with the ERIC-2 primer (5’-AAGTAAGTGACTGAGGGTGAGCG-3’) as described previously (12,13). Amplification was carried out by using the following program: 95°C for 5 min followed by 30 cycles of 30 s at 90°C, 1 min at 52°C, and 8 min at 72°C. The final extension step was performed at 72°C for 16 min. The analysis of amplified products was performed in 1.5% agarose gels. All samples were prepared and examined on at least three separate occasions. The PCR patterns were considered to be identical on the basis of similar numbers and matching positions of all major bands. Small differences in the intensities of faint bands were ignored.

Conjugation: Conjugation experiments were performed...
between *E. cloacae* isolates and *E. coli* JM109 as described previously (14). Transconjugants were selected on Luria-Bertani (LB) agar plates containing ampicillin and nalidixic acid (50 μg/ml each). All isolates were identified as *E. coli* by using an API20-E system, and the presence of plasmids in *E. cloacae* and transconjugant *e. coli* JM109 was confirmed by plasmid isolation.

**β-Lactamase assays and isoelectric focusing (IEF):** Cells were grown in LB broth with the appropriate antibiotic to the exponential phase and were harvested by centrifugation. The cell pellets were washed with 100 mM phosphate buffer saline solution (pH 7.0) and then were resuspended in the same buffer. Following disruption of the cells on ice with sonication, the cell lysate was centrifuged at 20,000 ×g for 90 min at 4°C and the β-lactamase-containing supernatant was retained. The enzymatic activity was determined spectrophotometrically at 25°C with a double-beam spectrophotometer at the appropriate wavelengths, and one unit of β-lactamase activity was defined as the amount of enzyme hydrolyzing 1 μmol of cephalothin per min per mg of protein at 37°C and pH 7.0 (14). The β-lactamases obtained were also analyzed by IEF at 10°C as described (15) using a slab of IEF gels (0.3 mm thickness) comprised of 5% (wt/vol) acrylamide, 2.6% (wt/vol) bisacrylamide, and 2% (wt/vol) ampholine (pH 3.5-9.5; Pharmacia, Buckinghamshire, UK). β-Lactamases were visualized using nitrocefin (about 1 mM) and the gradient markers of pI 4.65 - 9.60 (Bio-Rad Laboratories, Hercules, Calif., USA) were used as standards. Clavulanic acid and cloxacillin inhibiting tests were performed at the same time (16).

**PCR screening of the plasmids from ESBL-producing isolates of *E. cloacae* and transconjugants of *E. coli* and sequencing of the plasmid-borne *bla* genes:** Plasmid DNA was isolated by the alkaline lysis method from the ESBL-producing isolates of *E. cloacae* and transconjugants of *E. coli*. The presence of *bla* genes in the isolated plasmids was subsequently assayed by PCR using three pairs of primers designed from the sequences of the genes for SHV-type (GenBank accession no. X980101), TEM-type (GenBank accession no. X980112), and CTX-M-type (GenBank accession no. X980894) β-lactamases. These primers are as follows: SHV-type: 5'-CTGCGGATCCATGCGTTATATCGCTTG-3' (forward); 5'-CTCGGAATCTATGCGTTACGATGTC-3' (reverse); TEM-type: 5'-CTGCGGATCCATGCGTTATATCGCTTG-3' (forward); 5'-CTGCGGAATCTACGATGTC-3' (reverse); CTX-M-type: 5'-CTGCCGAGATCCAATGCTTAATCAGT-3' (forward); 5'-CTGCCGATCTTTAACACGTTGTTG-3' (reverse).

One of the two restriction endonuclease sites (i.e., BamHI or EcoRI as underlined) was added to the primers to facilitate subsequent cloning (8,10). The PCR mixtures were heated for 3 min at 94°C and then subjected to 30 cycles of 1 min each at 94°C, 55°C and 72°C with a final 7 min at 72°C. The products from each of several independent PCRs were purified and subsequently sequenced by Shanghai Jikang Biology Technology Co. Ltd., Shanghai, China. The nucleotide sequences and the deduced protein sequences were analyzed with the software available at the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

**RESULTS**

Resistance of ESBL-producing isolates: Based on the phenotype of resistance to oxyimino cephalosporins and aztreonam, 18 of 59 isolates were considered to be ESBL producers and further studied. The detailed antibiotic susceptibility of these isolates is presented in Table 1. The hydrolysis rates of cephalothin by the crude β-lactamases from these strains ranged from 1,282 to 2,135 nmol/min/mg (Table 2). The fact that most of them were more resistant to cefotaxime and aztreonam than to ceftazidime undermined the high prevalence of CTX-M-type ESBL.

**ERIC-PCR and conjugation:** The results from ERIC-PCR revealed that the 18 isolates comprised 7 distinct genotypes (Figure 1), suggesting that these ESBL producers were unlikely to have resulted from an outbreak of a single resistant strain. Plasmids were detected in all 18 clinical isolates, and successful transconjugation was also obtained with these isolates, with confirmation of the relevant plasmids in the transconjugants. The transconjugants obtained were resistant to penicillin and most of the cephalosporins tested, which clearly demonstrated that the plasmids contributed to the β-lactam resistance phenotype, which was similar to the results for the clinical isolates (these data are partially described in references 8 and 10).

**pl analysis:** All isolates showed at least one IEF band (range, one to three bands), and the appearance of all IEF bands was suppressed by the addition of clavulanic or cloxacillin. Among the 18 strains, 15 strains showed bands with a pl value of 5.4, 1 strain showed a band with a pl value of 7.6, and 16 strains showed bands with pl values of >8.0 (Table 2). IEF also confirmed that all the transconjugants carried the

<table>
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<th>β-lactam</th>
<th>Susceptible (no.)</th>
<th>Intermediate susceptible (no.)</th>
<th>Resistant (no.)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (μg/ml)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (μg/ml)</th>
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<td>0</td>
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<td>0.25</td>
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<sup>1</sup>: Clavulanic acid was used at a fixed concentration of 2 μg/ml.

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**Table 1. β-lactam susceptibility of ESBL-producing *E. cloacae* strains**
same β-lactamases (except the β-lactamase with a pI value of 8.3) as their parental strains. The activity of the β-lactamase with a pI of 8.3 was inhibited in the presence of cloxacillin, suggesting it is chromosomally encoded by class C β-lactamase (17).

Distribution of the genotypes of ESBL-producing strains:

Plasmids were isolated from 18 isolates and were used as templates for screening of the potential plasmid-borne bla genes by PCR with the primers for SHV, TEM or CTX-M type enzymes. The PCR results revealed that 3, 2, and 13 plasmids contained the genes, respectively, for SHV, TEM, and TEM and CTX-M type enzymes. The TEM enzymes included TEM-1, TEM-141, and both TEM and CTX-M type enzymes. Considering that plasmids contained the genes, respectively, for SHV, TEM, and TEM-157, while all of the CTX-M enzymes were CTX-M-22 (Table 2). The IEF assay also supported the presence of these enzymes when the following values of pIs were used as potential indicators of appropriate class A β-lactamases: SHV enzymes with a pI of 7.6-8.2, TEM enzymes with a pI of 5.4 and CTX-M enzymes with a pI of 8.7 (available online at http://www.lahey.org/Studies).

Nucleotide sequence accession no.: The following β-lactamase genes have been deposited to the GenBank nucleotide sequence database under the following accession nos.: AY956335 (TEM-141), DQ090059 (TEM-157), and DQ013287 (SHV-70). The sequences were also submitted to the database of “Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant β-Lactamases” at the Lahey Clinic, Burlington, Mass., USA (http://www.lahey.org/Studies).

**DISCUSSION**

Enterobacteriaceae carrying ESBLs or plasmid-mediated cephamycines have emerged as significant pathogens. However, since *E. cloacae* isolates typically produce inducible chromosomal β-lactamases, the spread of ESBL-producing *E. cloacae* isolates may be enhanced by underdetection and underreporting (18,19). Infections due to such strains are associated with prolonged hospital stays, increased healthcare costs, and increased mortality if appropriate therapy is delayed (18-20). Recently, a high prevalence of plasmid-encoded ESBL producers, including a surprisingly rapid increase in the recognition of *E. cloacae* clinical isolates containing CTX-M-type β-lactamases, has been observed in China (3,7,8,21), and has led to increasing concern over the treatment of clinical infections. Using PCR and IEF, we were here able to demonstrate that there are three types of plasmid-mediated ESBLs with the predominant CTX-M-22 enzymes. Intriguingly, the CTX-M-22 enzymes were shown to coexist with TEM-type enzymes in 13 of the isolates tested. It seemed that the co-linked bla genes constituted the genetic support of acquired *blaCTX-M* genes (3), such as an integration of the CTX-M-type gene into the TEM-type gene containing integron. In this survey, only one-ninth of isolates (2/18) were susceptible to clavulanic acid and cefoxitin. This resistance pattern could
be due to the presence of overproduced class C enzymes (1), which were found in some of our isolates. However, many inhibitor-resistant isolates might not overproduce this enzyme, which suggests that other mechanisms such as hyperproduction of ESBLs and altered outer membrane porins/drug efflux systems could be involved (22,23).

There are two main mechanisms for the spread of ESBLs: the dissemination of an ESBL-encoding plasmid among various clones or the spread of an ESBL-producing clone (2,20). In our study, ERIC-PCR typing revealed that the 18 isolates belonged to distinct clones, while plasmids were detected in all clinical isolates and transconjugants, and the conjugative plasmids were responsible for the production of ESBLs in these strains. Even without information of the plasmid profiles of the isolates or genetic information of the plasmids, the conjugation of the plasmids at high frequencies of approximately 10^{-3} - 10^{-5} (data not shown) would strongly suggest that the increase of ESBL-producing isolates was likely the result of plasmid transfer in this region, as it is known that genes encoding the CTX-M-type enzymes are often found in transferable plasmids and are readily transmissible by conjugation (3,24). Furthermore, the 18 clinical isolates were from urine, pus, and sputa, indicating that these were important sources of the spread. Given our current epidemiological data regarding ESBLs and given the fact that ESBL production is also relatively common among K. pneumoniae and E. coli (3,9), stricter measures will be required to control the further spread of these pathogens in hospital settings.

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