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Clinical Isolates of *Enterobacteriaceae* Producing Extended-Spectrum β-Lactamases: Prevalence of CTX-M-3 at a Hospital in China

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The prevalence of extended-spectrum β-lactamase-producing strains was demonstrated in 5 of 44 (11.4%) *Escherichia coli*, 17 of 43 (39.5%) *Klebsiella pneumoniae*, 3 of 50 (6.0%) *Enterobacter cloacae*, and 2 of 25 (8.0%) *Citrobacter freundii* strains at a teaching hospital in China. Nineteen of these 27 strains expressed CTX-M-3 β-lactamase (pI 8.6). A subset of the clinical isolates expressing the CTX-M-3 enzyme, tested by pulsed-field gel electrophoresis, revealed multiple clones. Five isolates expressed a novel enzyme, SHV-43 (pI 8.0), which had two substitutions (Leu113Phe and Thr149Ser) compared with SHV-1.

Since extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL-Ent) were first recognized in 1983 (9), more than 90 TEM-family extended-spectrum β-lactamases (ESBLs), 37 SHV types, 13 OXA types, and 20 CTX-M types, have now been identified, along with a few ESBLs of unknown parentage (http://www.lahey.org/studies/webt.htm). ESBL-Ent have been found worldwide, and because of the broad resistance to multiple agents seen in these isolates and their ability to disseminate widely in hospitals, they are a great therapeutic challenge (8, 18). Resistance to broad-spectrum β-lactams is becoming an ever-increasing problem in China (6, 23). In this study, we investigate the prevalence and genotypic characteristics of ESBL-Ent strains from Peking Union Medical College Hospital in China.

Forty-four isolates of *Escherichia coli*, 43 isolates of *Klebsiella pneumoniae*, 50 isolates of *Enterobacter cloacae*, and 25 isolates of *Citrobacter freundii* were sequentially and nonrepetitively collected from inpatients at Peking Union Medical College Hospital (a 1,000-bed tertiary-care hospital in Beijing) from February to May 1999. No temporal clustering of cases was noted except for six *K. pneumoniae* cases that occurred in a pediatric ward. The MICs of the antibiotics were determined by agar dilution methods established the National Committee for Clinical Laboratory Standards (NCCLS) (14). Antimicrobial standards were supplied by their corresponding manufacturers. *E. coli* strain ATCC 25922 and *Pseudomonas aeruginosa* strain ATCC 27853 were used as reference strains. ESBL-producing strains were identified by the ESBL Phenotypic Confirmatory Test according to NCCLS guidelines (15). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 (containing bla_{SHV-18}) were used as negative and positive controls, respectively.

Isoelectric focusing (IEF) was performed by the method of Matthew et al. (12) on polyacrylamide gels (pH 3.5 to 9.5; Amersham Pharmacia Biotech, Piscataway, N.J.). β-Lactamase extracts from strains known to produce TEM-1 (pI 5.4), TEM-10 (pI 5.6), SHV-12 (pI 8.2), or CMY-2 (pI 9.0) were used as IEF controls (Table 1). IEF standards were purchased from Bio-Rad (Hercules, Calif.). An IEF inhibition assay was performed by overlaying the gels with 250 μg of nitrocefin/ml with or without 0.3 mM cloxacillin or 0.3 mM clavulanic acid in 0.1 M phosphate buffer, pH 7.0 (22). Transfer of resistance was studied by performing conjugation experiments on a sample representative of 11 strains by using *E. coli* strain C600 (*lac* negative, Nal^r Rif^r) as the recipient. Transconjugants were selected on trypticase soy agar containing 10 μg of cefotaxime, 50 μg of nalidixic acid, and 60 μg of rifampin each per ml and reconfirmed by selection on MacConkey agar (Becton Dickinson) containing the same antibiotics.

Plasmid DNA was isolated with a plasmid miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. λ DNA/*Hin*dIII fragments were used to estimate plasmid size (Gibco BRL Life Technologies). A restriction enzyme (RE) digest was performed on four CTX-M-3-containing isolates from different medical wards and sources (CH7, CH11, CH13, and CH27) with the *Eco*RI or *Hpa*I REs (Gibco BRL Life Technologies).

The six *K. pneumoniae* isolates from an apparent cluster in a pediatric ward were subjected to pulsed-field gel electrophoresis (PFGE) typing performed by digesting chromosomal DNA with *Xba*I as previously described (13). Strain types were considered unique if there was a more than six-band difference (25). Plasmids from five of the six isolates (CH6, CH8, CH19, CH21T, and CH22) were subjected to RE digest.

***bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$.** β-Lactamase genes were amplified by PCR. Plasmid DNA from all transconjugants was used as a template in PCRs. The primers and PCR controls used are shown in Table 2. The strain containing the $bla_{CTX-M-5}$ gene was used as a positive control for the amplification of bla_{CTX-M} subgroup II. PCR products were purified by use of the QIAquick PCR purification kit (Qiagen). Direct cycle sequencing in both directions was performed with an automatic 373A DNA sequencer (Applied Biosystems, Foster City, Calif.) or with the AB Prism 377 DNA sequencer (PerkinElmer, Foster City, Calif.).

Five of the 44 (11.4%) strains of *E. coli*, 17 of the 43 (39.5%) strains of *K. pneumoniae*, 3 of the 50 (6.0%) strains of *E. clo-*

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TABLE 1. Bacterial strains used as IEF and PCR controls

Strain[a]	Type of β-lactamase	Origin[b]	Reference
E. coli DH5α/pBR322	TEM-1	USA	24
K. pneumoniae	TEM-10	USA	21
K. pneumoniae KPLA-1	SHV-12	Switzerland	16
E. coli J53-2-194	CMY-2	USA	7
E. coli DH5α/pCLL3417	CTX-M-5	Latvia	4

[a] KPLA-1(SHV-12), *E. coli* J53-2-194, and *E. coli*/pCLL3417 were generous gifts from H. Hächler, R. A. Bonomo, and P. A. Bradford, respectively.
[b] USA, United States of America.

acae, and 2 of the 25 (8.0%) strains of *C. freundii* presumably produced ESBLs. Characteristics of these 27 ESBL-Ent are shown in Table 3. Cefotaxime MICs were much higher than ceftazidime MICs for these strains. All of the strains were susceptible to imipenem except one strain of *E. cloacae*.

Nineteen of the 27 strains (70.3%) expressed β-lactamase with a pI of 8.6, and 5 strains expressed an enzyme with a pI of 8.0. Eighteen isolates also produced a β-lactamase with a pI of 5.4 that was consistent with TEM-1, and four isolates produced the enzyme with a pI of 7.6 that was consistent with SHV-1 (Table 3). An IEF inhibition assay performed on all strains showed that an enzyme with a pI of 7.9 in isolate CH23 was inhibited with cloxacillin, suggesting an AmpC-type enzyme, while others were inhibited with clavulanic acid, suggestive of non-AmpC enzymes (20). This enzyme with a pI of 7.9 was not transferred by conjugation. Cefotaxime resistance was much higher than ceftazidime resistance in all of the 11 transconjugants, which remained susceptible to cefoxitin (Table 3). A variety of plasmids ranging in size from 1 to 48 kb were visualized by electrophoresis (Table 3). RE analysis done on plasmids from four clinical strains from the medical ward carrying a single plasmid and harboring CTX-M-3 did not reveal an identical digestion pattern.

Three of the six isolates of *K. pneumoniae* in the pediatric ward, CH6, CH19, and CH21, belonged to the same PFGE type (type A), and all three carried the $bla_{\text{SHV-43}}$ gene expressing an enzyme with a pI of 8.0. RE digestion performed on the plasmid DNA of these three isolates indicated that CH6 and CH21T were identical. Three other isolates of *K. pneumoniae* from the same ward, CH8, CH17, and CH22, were all of unique PFGE types. These three isolates carried the $bla_{\text{CTX-M-3}}$ gene, producing an enzyme with a pI of 8.6. RE digestion performed on two of the three isolates indicated patterns different from each other and from the four isolates from the medical ward carrying the same $bla_{\text{CTX-M-3}}$ gene.

CTX-M-specific PCR performed on all transconjugants and three clinical strains (with a pI of 8.3) indicated that only those with a pI of 8.6 ($n = 8$) were positive for CTX-M-3-subgroup-specific PCR. Sequencing confirmed the presence of the $bla_{\text{CTX-M-3}}$ gene in these isolates (Table 3). SHV-specific PCR was positive for five strains with a pI of 8.0, and two of these were sequenced (CH6 and CH20). The deduced amino acid sequence had two substitutions compared with that for SHV-1: phenylalanine for leucine at position 113 (codon change of CTT to TTT) and serine for threonine at position 149 (codon change of ACT to TCT). The novel enzyme was designated SHV-43. Sequencing performed on the transconjugants carrying enzymes with a pI of 5.4 (CH2T, CH15T, CH25T, and CH26T) or 7.6 (CH8T) indicated the presence of the $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$ genes, respectively.

This study confirmed the presence of ESBLs in *E. cloacae* and *C. freundii* by using clavulanic acid. Due to clavulanic acid usually inducing β-lactam resistance and the overproduction of functional group 1 β-lactamases in these strains (5), the prev-

TABLE 2. Nucleotide sequences of the oligonucleotides used for PCR amplification and DNA sequencing

Gene and primer	Nucleotide sequence (position)[d]	Reference or source
bla_{TEM}[a]		
JP2	5′-TTG AAG ACG AAA GGG CCT CGT G-3′ (on promoter 4–25)	24
BLA30	5′-CTG ACG CTC AGT GGA ACG-3′ (nt 3147–3165 position corresponding to pBR322)[e]	24
BLA34	5′-GGG GCC AGA TGG TAA GCC C-3′ (nt 949–968)	24
bla_{SHV}[b]		
SHVA	5′-TGG TTA TGC GTT ATA TTC GCC-3′ (nt 120–140)	17
SHVB	5′-GGT TAG CGT TGC CAG TGC T-3′ (nt 990–972)	17
SHVC	5′-ATC ATG GGA AAG CGT TCA TC-3′ (nt 318–299)	This study
SHVD	5′-TTG ATC CGC TCC GTG CTG-3′ (nt 773–790)	This study
$bla_{\text{CTX-M}}$[c]		
P1	5′-ATG GTT AAA AAA TCA CTG CGC C-3′ (Y10278; nt 1–22)	This study
P3	5′-ATG ATG ACT CAG AGC ATT CG-3′ (Y14156; nt 1–20)	This study
P4	5′-CGG CCT GTA TTT CGC TGT TG-3′ (AF189721; nt 314–333)	This study
P2b	5′-TCC CGA CGG CTT TCC GCC TT-3′ (AJ005044; nt 833–814)	This study

[a] JP2 and BLA30 (downstream of the 3′ end of the *bla* gene) were used to amplify the bla_{TEM} genes; JP2, BLA30, and BLA34 were used to sequence the amplified bla_{TEM} genes. The *E. coli*/pBR322(TEM-1) strain was used as the TEM-specific PCR positive control.
[b] SHVA and SHVB were used to amplify the bla_{SHV} genes; SHVA, SHVB, SHVC, and SHVD were used to sequence the entire range of amplified bla_{SHV} genes. The KPLA-1(SHV-12) strain was used as the SHV-specific PCR positive control.
[c] P1 and P2b were used to amplify $bla_{\text{CTX-M}}$ subgroup I genes (including $bla_{\text{CTX-M-1}}$ $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-10}}$); P3 and P2b were used to amplify $bla_{\text{CTX-M}}$ subgroup II genes (including $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-4}}$, $bla_{\text{CTX-M-5}}$, $bla_{\text{CTX-M-6}}$, and $bla_{\text{CTX-M-7}}$); and P4 and P2b were used to amplify $bla_{\text{CTX-M}}$ subgroup III genes (including $bla_{\text{CTX-M-8}}$). Sequencing used the same primers as those for PCR. *E. coli*/pCLL3417(CTX-M-5) was used for the positive control for PCR of subgroup II and the negative control for the other subgroup.
[d] nt, nucleotide.
[e] Downstream of the 3′ end of the *bla* gene.

TABLE 3. Characteristics of 27 ESBL-producing strains and 11 transconjugants

Isolate no.[a]	Ward[b]	Source	Organism[c]	MIC (μg/ml)[d]					Zone diam[e] of FOX (mm)	pI(s) of β-lactamase(s)	β-Lactamase(s)	Plasmid(s) (kb)
				CTX	CTX/cla	CAZ	CAZ/cla	IMP				
CH1	MW1	Sputum	eco	64	1	16	8	1	6	5.4, 7.6, 8.3	TEM-1, SHV-1, SHV-like	47, 7, 5, 2
CH1T	MW1	Sputum	eco	64	0.5	4	0.5	0.25	30	5.4, 7.6, 8.3	TEM-1, SHV-1, SHV-like	47
CH2	SW1	Urine	eco	>256	0.50	16	2	1	18	5.4, 8.6	TEM-1, CTX-M-3	42, 20, 2
CH2T	SW1	Urine	eco	>256	0.25	16	2	0.25	24	5.4, 8.6	TEM-1, CTX-M-3	42, 2
CH3	SW1	Pelvic drainage	eco	16	0.13	4	0.5	0.25	23	5.4, 8.6	TEM-1, CTX-M-3	37, 31
CH4	MW2	Urine	eco	>256	0.25	1	0.5	0.25	19	5.4, 8.0	TEM-1, SHV-43	42, 20, 1
CH5	ICU	Ascites	eco	>256	0.13	2	0.25	0.5	23	5.4, 8.6	TEM-1, CTX-M-3	42, 22
CH6	PW	Blood	kpn	>256	0.06	2	0.5	0.25	27	8.0	SHV-43	32
CH7	MW2	Blood	kpn	>256	0.06	1	0.25	0.25	28	5.4, 8.6	TEM-1, CTX-M-3	37
CH8	PW	Catheter blood	kpn	>256	0.06	4	0.25	0.25	27	5.4, 5.9, 7.6, 8.6	TEM-1, TEM-like, SHV-1, CTX-M-3	39
CH8T	PW	Catheter blood	kpn	256	0.25	4	0.5	0.25	23	5.4, 5.9, 7.6, 8.6	TEM-1, TEM-like, SHV-1, CTX-M-3	39
CH9	GW	Genital	kpn	64	0.02	1	0.25	0.5	25	5.4, 8.6	CTX-M-3	31, 9, 4, 3
CH10	SW2	Sputum	kpn	64	0.06	1	0.25	0.5	25	7.6, 8.3	SHV-1, SHV-like	39, 13
CH10T	SW2	Sputum	kpn	128	0.25	2	0.75	0.25	23	7.6, 8.3	SHV-1, SHV-like	39, 13
CH11	MW3	Sputum	kpn	>256	0.03	1	0.25	0.5	25	8.6	CTX-M-3	37
CH12	SW2	Sputum	kpn	>256	0.06	4	0.5	0.5	26	7.6, 8.3	SHV-1, SHV-like	43
CH13	MW4	Sputum	kpn	32	0.06	2	0.25	0.5	26	8.6	CTX-M-3	30
CH13T	MW4	Sputum	kpn	64	0.13	2	0.25	0.25	24	8.6	CTX-M-3	30
CH14	MW5	Sputum	kpn	32	0.06	2	0.25	0.25	22	5.4, 8.6	TEM-1, CTX-M-3	32, 4, 3, 1
CH15	ICU	Sputum	kpn	16	0.13	4	1	0.5	23	5.4, 8.6	TEM-1, CTX-M-3	37, 31, 3
CH15T	ICU	Sputum	kpn	16	0.13	2	1	0.25	23	5.4, 8.6	TEM-1, CTX-M-3	37
CH16	ICU	Pelvic drainage	kpn	>256	0.03	1	0.5	0.5	25	5.4, 8.6	TEM-1, CTX-M-3	43, 28, 4, 3, 1
CH17	PW	Catheter tip	kpn	>256	0.06	2	0.25	0.5	26	5.4, 8.6	TEM-1, CTX-M-3	37, 34, 2
CH17T	PW	Catheter tip	kpn	>256	0.13	2	0.25	0.25	23	5.4, 8.6	TEM-1, CTX-M-3	37
CH18	ICU	Abdominal drainage	kpn	>256	0.06	1	0.25	0.25	26	5.4, 8.6	TEM-1, CTX-M-3	31, 9, 4, 3
CH19	PW	Intubation	kpn	>256	0.06	4	0.5	0.5	26	8.0	SHV-43	32
CH20	MW5	Ascites	kpn	>256	0.06	1	0.25	0.5	26	8.0	SHV-43	32
CH21	PW	Umbilical swab	kpn	16	0.06	2	0.5	0.5	26	8.0	SHV-43	48, 32
CH21T	PW	Umbilical swab	kpn	4	0.13	1	0.5	0.25	29	8.0	SHV-43	32
CH22	PW	Wound	kpn	>256	0.06	2	0.25	0.5	26	5.4, 8.6	TEM-1, CTX-M-3	39
CH23	SW2	Sputum	ecl	>256	32	>256	16	32	6	5.4, 7.9, 8.6	TEM-1, AmpC-like, CTX-M-3	37, 2, 1
CH23T	SW2	Sputum	ecl	>256	0.25	32	2	0.5	23	5.4, 8.6	TEM-1, CTX-M-3	37, 1
CH24	SW3	Abdominal drainage	ecl	128	16	4	4	4	6	5.4, 8.6	TEM-1, CTX-M-3	37, 3
CH25	SW1	Sputum	ecl	>256	1	>256	8	0.5	6	5.4, 8.6	TEM-1, CTX-M-3	38, 12, 8, 3, 1
CH25T	SW1	Sputum	ecl	64	0.13	32	1	0.25	22	5.4, 8.6	TEM-1, CTX-M-3	38, 3, 1
CH26	MW3	Sputum	cfr	>256	0.25	32	2	0.5	15	5.4, 8.6	TEM-1, CTX-M-3	33, 15, 2, 1
CH26T	MW3	Sputum	cfr	64	0.13	4	1	0.25	25	5.4, 8.6	TEM-1, CTX-M-3	33
CH27	MW6	Throat swab	cfr	>256	0.13	>256	0.5	0.5	28	8.6	CTX-M-3	32
EC C600			eco	0.125	0.13	0.5	0.5	0.25	23			

[a] EC C600, recipient; T, transconjugant.
[b] MW, medical ward; SW, surgical ward; PW, pediatric ward; ICU, intensive care unit.
[c] eco, *E. coli*; kpn, *K. pneumoniae*; ecl, *E. cloacae*; cfr, *C. freundii.*
[d] CTX, cefotaxime; CTX/cla, cefotaxime-clavulanic acid; CAZ, ceftazidime; CAZ/cla, ceftazidime-clavulanic acid; IMP, imipenem.
[e] FOX, cefoxitin. NCCLS breakpoints: resistant, ≤14 mm; susceptible, ≥18 mm.

alence of ESBLs may be underestimated (10, 26). The methods to detect ESBLs in *Enterobacter* and *Citrobacter* strains are unavailable in NCCLS guidelines.

Unlike the United States, CTX-M β-lactamase was the most prevalent in this hospital. Class A plasmid-mediated CTX-M β-lactamase constitutes one of the minor families of ESBLs that are much more active against cefotaxime than ceftazidime. In 1990, the first CTX-M enzyme (CTX-M-1) was reported in *E. coli* in Germany (2). To date, 20 members of this group have been reported in the world. They are not related to the TEM or SHV β-lactamases but show homology to the chromosomal β-lactamases of *Klebsiella oxytoca* and *Citrobacter diversus* (3, 19) and even greater homology to the chromosomal gene of *Kluyvera ascorbata* (1a). CTX-M-producing strains have now been reported over a wide geographic area including the Middle and Far East, South America, and Europe (1, 3, 4, 11).

It has been demonstrated that ESBLs arise because of point mutations occurring in the face of selective pressure due to the use of extended-spectrum cephalosporins. In our hospital, from 1995 to 1999, the use of cefotaxime increased from 19 to 102 kg of body weight/year while the use of ceftazidime increased from 9 to 15 kg/year. Presumably, the selective pressure of cefotaxime is responsible for the selection of cefotaxime-hydrolyzing enzymes. Our colleague also found CTX-M-11 in one strain of *K. pneumoniae* at our hospital in 2000 (X. Zhu, personal communication) (accession no. AY005110). This enzyme differs from CTX-M-3 by three amino acid changes. This study also found that a clonal (SHV-43 ESBL) spread occurred in the pediatric ward during the study period.

Nucleotide sequence accession number. The nucleotide sequence data for SHV-43 reported appear in the GenBank nucleotide sequence database under accession no. AY065991.

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