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Susceptibility of Extended-Spectrum-β-Lactamase-Producing *Enterobacteriaceae* According to the New CLSI Breakpoints[▽]

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In 2010 the Clinical and Laboratory Standards Institute (CLSI) lowered the susceptibility breakpoints of some cephalosporins and aztreonam for *Enterobacteriaceae* and eliminated the need to perform screening for extended-spectrum β-lactamases (ESBLs) and confirmatory tests. The aim of this study was to determine how many ESBL-producing strains of three common species of *Enterobacteriaceae* test susceptible using the new breakpoints. As determined with the CLSI screening and confirmatory tests, 382 consecutive ESBL-producing strains were collected at Huashan Hospital between 2007 and 2008, including 158 strains of *Escherichia coli*, 164 of *Klebsiella pneumoniae*, and 60 of *Proteus mirabilis*. Susceptibility was determined by the CLSI agar dilution method. CTX-M-, TEM-, and SHV-specific genes were determined by PCR amplification and sequencing. $bla_{\text{CTX-M}}$ genes alone or in combination with bla_{SHV} were present in 92.7% (354/382) of these ESBL-producing strains. Forty-two (25.6%) strains of *K. pneumoniae* harbored SHV-type ESBLs alone or in combination. No TEM ESBLs were found. Utilizing the new breakpoints, all 382 strains were resistant to cefazolin, cefotaxime, and ceftriaxone, while 85.0 to 96.7% of *P. mirabilis* strains tested susceptible to ceftazidime, cefepime, and aztreonam, 41.8 to 45.6% of *E. coli* strains appeared to be susceptible to ceftazidime and cefepime, and 20.1% of *K. pneumoniae* were susceptible to cefepime. In conclusion, all ESBL-producing strains of *Enterobacteriaceae* would be reported to be resistant to cefazolin, cefotaxime, and ceftriaxone by using the new CLSI breakpoints, but a substantial number of ESBL-containing *P. mirabilis* and *E. coli* strains would be reported to be susceptible to ceftazidime, cefepime, and aztreonam, which is likely due to the high prevalence of CTX-M type ESBLs.

Enterobacteriaceae, especially those producing extended-spectrum β-lactamases (ESBLs), can cause various nosocomially acquired infections (14, 17). A much higher incidence of ESBLs in *Escherichia coli* and *Klebsiella pneumoniae* has been reported in China than in most other regions of the world (10, 16). The CHINET national bacterial surveillance project found that 56% of *E. coli* strains and 41% of *K. pneumoniae* strains were ESBL producers in 2009 (31). Some ESBL-producing organisms appeared to be susceptible to β-lactam antibiotics with former breakpoints but failed to respond when such drugs were used in therapy; therefore, the Clinical and Laboratory Standards Institute (CLSI) recommended performance of ESBL screening and confirmatory tests and a report of resistance to penicillins, cephalosporins, and aztreonam if the ESBL confirmatory tests were positive (7).

The confirmatory test method, however, is established only in *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *Proteus mirabilis* strains and has not been standardized for other Gram-negative bacilli. In addition, strains simultaneously producing both ESBLs and AmpC-type enzymes may give a positive ESBL screening result but have a false-negative confirmatory test result (13, 20). In 2010, CLSI lowered the susceptibility breakpoints of some cephalosporins and aztreonam for *Enterobacteriaceae* and removed the need to perform ESBL screening and confirmatory tests except for epidemiological or infection control purposes. The susceptibility breakpoints for cefazolin, cefotaxime, ceftizoxime, and ceftriaxone were modified from 8 μg/ml to 1 μg/ml; and for ceftazidime and aztreonam they were modified from 8 μg/ml to 4 μg/ml (8).

The CTX-M β-lactamases are the most prevalent ESBLs in China, and a variety of CTX-M enzymes have been observed, including CTX-M-1, -3, -9, -13, -14, and -15 (16, 33, 34). CTX-M ESBLs are also the most common ESBLs worldwide, and the majority of *Enterobacteriaceae* isolates predominantly produced CTX-M-15, belonging to the CTX-M-1 group of ESBLs, and/or CTX-M-14, belonging to the CTX-M-9 group of ESBLs (4, 10, 11). Among 552 ESBL-producing strains isolated in Canada, CTX-M-14 was more common than CTX-M-15 (211 versus 128 strains) (27). Members of the CTX-M-1 group, particularly CTX-M-15, are dominant ESBLs in nearly all European countries. Of 181 ESBL-producing *E. coli* strains collected in Portugal, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ were harbored in 109 and 9 strains, respectively (19).

Different ESBL types vary in their ability to hydrolyze cephalosporins and aztreonam. CTX-M β-lactamases hydrolyze cefotaxime and ceftriaxone better than they do ceftazidime (22, 24), while ceftazidime is usually the best substrate for SHV ESBLs (2, 3). Furthermore, most CTX-M-1-group enzymes have more hydrolytic activity against ceftazidime, cefepime,

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TABLE 1. Comparison of prevalence of CTX-M-1-group and CTX-M-9-group ESBL genes in 382 ESBL-producing strains

Strain	Rate of detection (%)[a]				
	CTX-M-1 group alone	CTX-M-9 group alone	CTX-M-1 group + CTX-M-9 group	CTX-M-9 group + CTX-M-25 group	Total[c]
E. coli	25.3 (40/158)	62.7 (99/158)	8.2 (13/158)	1.9 (3/158)	98.1 (155/158)
K. pneumoniae[b]	40.9 (67/164)	18.3 (30/164)	7.3 (12/164)	0	66.5 (109/164)
P. mirabilis	5.0 (3/60)	85.0 (51/60)	6.7 (4/60)	0	96.7 (58/60)

[a] Data in parentheses represent the number of isolates positive for the gene/total number of isolates tested.
[b] Strains carrying SHV-type genes are not listed. SHV genes were present alone in 7 (4.3%) of 164 *K. pneumoniae* strains and in combination with CTX-M genes in 34 (20.7%) of 164 *K. pneumoniae* strains; no SHV genes were detected in *E. coli* and *P. mirabilis* strains.
[c] Genotypes could not be determined in 3 strains of *E. coli*, 14 of *K. pneumoniae*, and 2 of *P. mirabilis*.

and aztreonam than do enzymes of the CTX-M-9 group (3, 22, 26).

Preliminary reports suggest that the revised breakpoints may not detect all ESBL-producing strains (29, 30). Our aim in this study was to determine how many ESBL-producing strains among three common species of *Enterobacteriaceae* collected from Huashan Hospital test susceptible to five cephalosporins and aztreonam by using the new breakpoints.

MATERIALS AND METHODS

Bacterial strains. As determined with the previously recommended CLSI disk diffusion ESBL screening and confirmatory test (7), 382 consecutive and nonduplicate ESBL-producing strains were collected at Huashan Hospital, including 158 strains of *E. coli* and 164 of *K. pneumoniae* in 2008 and 60 of *P. mirabilis* between 2007 and 2008.

PCR detection and sequencing of ESBL genes. CTX-M-, TEM-, and SHV-specific genes were determined by PCR with the primers reported previously (2, 9, 32), and the PCR products of TEM- and SHV-specific genes were sequenced. The nucleotide sequences were analyzed with the BLAST program (http://www.ncbi.nlm.nih.gov/blast).

Susceptibility testing. MICs of cefazolin, cefotaxime, ceftriaxone, ceftazidime, cefepime, and aztreonam were measured by the agar dilution method according to the recommendations of the CLSI (7). Results were interpreted according to the new breakpoints established by the CLSI (8). The control strains used for this study were susceptible strain *E. coli* ATCC 25922 and ESBL-producing strain *K. pneumoniae* ATCC 700603. The Etest (AB Biodisk, Solna, Sweden) was used to confirm the cefepime and aztreonam MIC results for *P. mirabilis* strains with MICs of ≤0.125 μg/ml to those two drugs when they were tested by the agar dilution method.

Statistical analysis. CTX-M-1-group and CTX-M-9-group occurrence rates in different species and the antimicrobial susceptibilities were compared by the Pearson chi-square test.

Nucleotide sequence accession number. The complete nucleotide sequence of TEM-183 has been submitted to GenBank and assigned accession number HQ529916.

RESULTS

ESBL genotypes. Of 382 strains with ESBL phenotypes, genotypes could be determined in 363 strains, but not in 3 strains of *E. coli*, 14 of *K. pneumoniae*, and 2 of *P. mirabilis*.

(i) Prevalence of $bla_{\text{CTX-M}}$ and bla_{SHV} ESBL genes. $bla_{\text{CTX-M}}$ genes were present alone or in combination with bla_{SHV} in 92.7% (354/382) of these ESBL-producing strains. $bla_{\text{CTX-M-1}}$-group ESBL genes were found in more than 60% of *K. pneumoniae* strains, a rate significantly higher than that for the $bla_{\text{CTX-M-9}}$ group ($P < 0.01$). $bla_{\text{CTX-M-9}}$-group ESBLs were found in more than 70% of *E. coli* strains and over 90% of *P. mirabilis* strains, rates significantly higher than the rate for the $bla_{\text{CTX-M-1}}$ group ($P < 0.01$). The CTX-M-2 and CTX-M-8 groups were not detected (Table 1).

Forty-one (25.0%) strains of *K. pneumoniae* harbored SHV-type ESBLs alone or in combination with CTX-M: SHV-12 ($n = 29$), SHV-2 ($n = 10$), or SHV-31 ($n = 2$). bla_{SHV} ESBL genes were not detected in any of the *E. coli* or *P. mirabilis* strains.

(ii) Prevalence of non-ESBL genes. Among 382 strains, 99 strains were positive for non-ESBL SHV types: $bla_{\text{SHV-11}}$ ($n = 64$), $bla_{\text{SHV-1}}$ ($n = 12$), $bla_{\text{SHV-61}}$ ($n = 15$), $bla_{\text{SHV-28}}$ ($n = 6$), and $bla_{\text{SHV-26}}$ ($n = 2$). bla_{TEM} genes were amplified from 263 of 382 strains. All bla_{TEM} genes belonged to the non-ESBL group, including TEM-1 in 259 strains, TEM-135 in 2 strains, and TEM-40 in 1 strain. A new TEM subtype that differed from TEM-1 by a Phe-to-Leu replacement at position 230 (T to C at nucleotide position 682) was cloned in a *K. pneumoniae* isolate and designated $bla_{\text{TEM-183}}$. A transformant with the cloned $bla_{\text{TEM-183}}$ gene did not have an ESBL phenotype.

(iii) Distribution of ESBL genes in bacterial strains. Only CTX-M ESBL genes were present in 98.1% of *E. coli* strains and 96.7% of *P. mirabilis* strains (Table 1), while both CTX-M and SHV ESBL genes were detected *in K. pneumoniae* strains: CTX-M alone in 66.5% (109/164), SHV alone in 4.3% (7/164), and both together in 20.7% (34/164).

Susceptibility testing. Using the new breakpoints, all 382 strains were resistant to cefazolin, cefotaxime, and ceftriaxone, while 91.7%, 85.0%, and 96.7% of the *P. mirabilis* strains tested susceptible to ceftazidime, cefepime, and aztreonam, respectively. Three strains had aztreonam MICs of 0.04 μg/ml, and 11 strains had aztreonam MICs of 0.06 μg/ml, as determined by Etest. A total of 45.6% and 41.8% of *E. coli* strains appeared to be susceptible to ceftazidime and cefepime, and 20.1% of *K. pneumoniae* strains were susceptible to cefepime (Table 2).

The relationship between ESBL production and antimicrobial susceptibility is shown in greater detail in Table 3. CTX-M-1 *E. coli* strains were more resistant to ceftazidime, cefepime, and aztreonam than CTX-M-9-group strains. Of 99 CTX-M-9-group *E. coli* strains, 22 strains were susceptible to the above three antibiotics and 11 were susceptible to ceftazidime and cefepime but intermediate to aztreonam, while among 40 CTX-M-1 strains, 20 were resistant to those three antibiotics and 8 were resistant to ceftazidime and aztreonam but intermediate to cefepime. β-Lactam resistance rates were much higher among *K. pneumoniae* strains than *E. coli* strains,

TABLE 2. Susceptibilities of six antimicrobial agents against 382 ESBL-producing strains of the *Enterobacteriaceae*

Bacterium	Antibiotic	No. of strains with MIC (μg/ml) of[a]:													MIC (μg/ml)		Susceptibility rate (%)
		0.04[b]	0.06[b]	0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	50%	90%	
E. coli (n = 158)	Cefotaxime	0	0	0	0	0	0	0	1	6	9	23	31	88	128	>128	0
	Ceftriaxone	0	0	0	0	0	0	0	0	1	5	13	25	114	128	>128	0
	Ceftazidime	0	0	0	1	4	22	30	15	21	19	23	12	11	8	64	45.6 (72/158)[c]
	Cefepime	0	0	0	0	1	5	13	17	30	35	25	24	8	16	64	41.8 (66/158)
	Aztreonam	0	0	0	1	0	3	4	15	17	28	30	24	36	32	>128	14.6 (23/158)
K. pneumoniae (n = 164)	Cefotaxime	0	0	0	0	0	0	0	2	7	4	10	24	117	>128	>128	0
	Ceftriaxone	0	0	0	0	0	0	0	0	1	4	10	15	134	>128	>128	0
	Ceftazidime	0	0	0	0	0	2	9	3	10	4	16	22	98	128	>128	8.5 (14/164)
	Cefepime	0	0	0	0	0	2	6	8	17	33	67	23	8	32	64	20.1 (33/164)
	Aztreonam	0	0	0	0	0	0	1	1	9	5	11	13	124	>128	>128	1.2 (2/164)
P. mirabilis (n = 60)	Cefotaxime	0	0	0	0	0	0	1	0	3	33	21	0	2	16	32	0
	Ceftriaxone	0	0	0	0	0	0	1	5	25	22	6	0	1	8	32	0
	Ceftazidime	0	0	14	19	12	6	1	3	3	0	1	0	1	0.25	4	91.7 (55/60)
	Cefepime	0	0	1	0	0	0	1	12	37	8	1	0	0	8	16	85.0 (51/60)
	Aztreonam	3	11	1	12	15	11	5	0	0	1	0	1	0	0.5	2	96.7 (58/60)

[a] All isolates had MICs of ≥128 μg/ml for cefazolin.
[b] Tested by Etest.
[c] Data in parentheses represent the number of isolates susceptible/total number of isolates tested.

but the data still showed that CTX-M-1-group strains were more resistant than CTX-M-9-group strains.

For 290 strains carrying CTX-M-1 or CTX-M-9 alone, the susceptibility rates for aztreonam, cefepime, and ceftazidime were 41% to 68% for CTX-M-9-group organisms but only 3 to 14% for CTX-M-1-group strains (Table 4).

DISCUSSION

In this study, we found that ESBL-producing strains of *Enterobacteriaceae* showed different patterns of resistance to various cephalosporins and aztreonam when using the new CLSI susceptibility breakpoints. All strains were resistant to cefazolin, cefotaxime, and ceftriaxone, but a substantial number of *P. mirabilis* and *E. coli* strains would be reported to be susceptible to ceftazidime, cefepime, and aztreonam. This may reflect the different abilities of ESBLs to hydrolyze different cephalosporins and aztreonam. $bla_{\text{CTX-M}}$ genes alone or in combination with bla_{SHV} were present in 93% of this set of ESBL-producing *Enterobacteriaceae* strains. In regions where CTX-M ESBLs are predominant, resistance to cefotaxime and ceftriaxone may be a better marker for ESBL presence than resistance to ceftazidime, cefepime, and aztreonam.

The susceptibilities of ESBL-producing *K. pneumoniae*, *E. coli*, and *P. mirabilis* strains to ceftazidime, cefepime, and aztreonam varied considerably. Surprisingly, 85% to 97% of ESBL-producing *P. mirabilis* strains appeared to be susceptible to ceftazidime, cefepime, and aztreonam. The variety profiles of susceptibility to the 3 drugs for all organisms might be due to MICs for many isolates falling close to the breakpoint. This variety may be related to differences in β-lactamase expression or outer membrane permeability. For example, decreased expression of outer membrane porins that are channels for β-lactam entry can considerably augment β-lactamase protection (1, 12, 18). In addition, *P. mirabilis*, which lacks intrinsic chromosomal β-lactamase genes, is entirely dependent upon acquisition of different β-lactamase genes to express a β-lactamase-mediated resistance phenotype (6, 17).

We also observed that *K. pneumoniae* was more resistant to ceftazidime, cefepime, and aztreonam than *E. coli*. This may relate to the significantly different distribution of the ESBL genotypes between *K. pneumoniae* and *E. coli*. At least 41 (25%) strains of *K. pneumoniae* carried SHV-type ESBLs alone or in combination, but none were found in *E. coli* strains. All 7 strains of *K. pneumoniae* with SHV ESBLs alone were resistant to ceftazidime and aztreonam, with MICs ranging from 32 to >128 μg/ml, and had cefepime MIC ranges of 2 to 16 μg/ml. In addition, the CTX-M-1-group ESBLs, which have good hydrolytic activity for ceftazidime, cefepime, and aztreonam, predominated in *K. pneumoniae*, while the CTX-M-9 group was preponderant in *E. coli*. The susceptibility to ceftazidime, cefepime, and aztreonam was significantly higher for the CTX-M-9-group than CTX-M-1-group strains.

Drawbacks of this study are that the *Enterobacteriaceae* strains were collected from one hospital, although each strain was isolated from a separate patient. However, most of the strains were found to produce CTX-M-type ESBLs in our study. Thus, this study could provide further understanding of CTX-M-type ESBLs, the most common ESBL worldwide.

At present, a few clinical trials have shown efficacy for extended-spectrum cephalosporins in patients infected with ESBL-producing isolates, but the number of patients studied is small (5, 15, 23). In a recent study (28), nine patients with ESBL-producing *Enterobacter cloacae* bloodstream infections were treated with cefepime, and it was found that four (66.7%) of six patients who were treated with cefepime and whose isolates had cefepime MICs of 0.25 to 3 μg/ml showed clinical failure. Further well-designed prospective clinical studies are needed to determine the efficacy of ceftazidime, cefepime, and aztreonam in the treatment of in-

TABLE 3. Relationship between ESBL production and antimicrobial susceptibility

Bacterium[a]	ESBL genotype (no. of isolates)	No. of isolates with the indicated susceptibility pattern	Cephalosporin susceptibility pattern[b]		
			Ceftazidime	Cefepime	Aztreonam
E. coli (n = 155)	CTX-M-9 group (99)	22	S	S	S
		13	R	R	R
		12	S	S	R
		11	I	R	R
		11	S	S	I
		10	S	I	R
		5	S	I	I
		4	R	S	R
		3	S	R	R
		3	I	I	R
		3	I	S	R
		2	R	I	R
	CTX-M-1 group (40)	20	R	R	R
		8	R	I	R
		4	S	S	R
		4	R	S	R
		1	I	I	R
		1	I	S	I
		1	I	S	R
		1	S	I	R
	CTX-M-1 group + CTX-M-9 group (13)	5	R	R	R
		3	R	I	R
		2	S	I	R
		1	R	S	R
		1	S	S	R
		1	I	R	R
	CTX-M-9 group + CTX-M-25 group (3)	2	R	R	R
		1	S	S	S
K. pneumoniae (n = 150)	CTX-M-1 group (67)	57	R	R	R
		6	R	I	R
		1	R	S	R
		1	S	S	I
		1	I	R	R
		1	S	S	S
	CTX-M-9 group (30)	7	R	I	R
		6	S	S	I
		4	R	R	R
		3	I	R	R
		3	R	S	R
		2	I	I	R
		2	S	S	R
		1	S	I	R
		1	I	S	I
		1	S	S	S
	CTX-M-1 group + SHV (16)	13	R	R	R
		1	R	I	R
		1	R	S	R
		1	S	S	I
	CTX-M-9 group + SHV (13)	9	R	I	R
		2	R	R	R
		2	R	S	R
	CTX-M-1 group + CTX-M-9 group (12)	11	R	R	R
		1	I	S	R
	SHV alone (7)	5	R	S	R
		2	R	I	R
	CTX-M-1 group + CTX-M-9 group + SHV (5)	3	R	R	R
		2	R	I	R
P. mirabilis (n = 58)	CTX-M-9 group (51)	44	S	S	S
		5	S	I	S
		1	S	R	S
		1	I	I	S
	CTX-M-1 group (3)	2	S	S	S
		1	R	I	R
	CTX-M-1 group CTX-M-9 group (4)	2	S	S	S
		2	I	S	S

[a] Genotypes could not be determined in 3 strains of *E. coli*, 14 of *K. pneumoniae*, and 2 of *P. mirabilis*.
[b] S, susceptible; R, resistant; I, intermediate.

TABLE 4. Comparison of rates of susceptibility to ceftazidime, cefepime, and aztreonam between CTX-M-1-group and CTX-M-9-group strains

Antibiotic	% susceptible[a]		*P* value
	CTX-M-1 group alone	CTX-M-9 group alone	
Ceftazidime	8.2 (9/110)	68.3 (123/180)	<0.01
Cefepime	13.6 (15/110)	60.6 (109/180)	<0.01
Aztreonam	2.7 (3/110)	41.1 (74/180)	<0.01

[a] Data in parentheses represent the number of isolates susceptible/total number of isolates tested.

fections caused by apparently susceptible ESBL-producing strains of the *Enterobacteriaceae*, especially in treating serious infections such as bacteremia.

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