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Complexity of *Klebsiella pneumoniae* Isolates Resistant to Both Cephamycins and Extended-Spectrum Cephalosporins at a Teaching Hospital in Taiwan

Jing-Jou Yan,[1] Wen-Chien Ko,[2] Hsiu-Mei Wu,[3] Shu-Huei Tsai,[1] Chin-Luan Chuang,[1] and Jiunn-Jong Wu[3]*

Department of Pathology,[1] Internal Medicine,[2] and Medical Technology,[3] College of Medicine, National Cheng Kung University, Tainan, Taiwan

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Among 99 clinical *Klebsiella pneumoniae* isolates resistant to cefoxitin and extended-spectrum cephalosporins, coexistence of AmpC (DHA-1, CMY-2, or CMY-8) and extended-spectrum β-lactamases (CTX-M and/or SHV) was detected in a total of 35. The remainder produced AmpC ($n = 42$), extended-spectrum β-lactamases ($n = 9$), metallo-β-lactamases ($n = 2$), or none of these enzymes ($n = 11$). Phenotypic characteristics of these isolates were demonstrated.

The majority of β-lactamases that confer resistance to extended-spectrum cephalosporins on *Klebsiella pneumoniae* are Ambler's molecular class A extended-spectrum β-lactamases (ESBLs) (3). β-Lactamases of molecular classes B and C, also termed metallo-β-lactamases (MBLs) and AmpC enzymes, respectively, provide a broader spectrum of resistance than ESBLs and have also been identified in *K. pneumoniae* (4, 6, 11). Unlike ESBLs, AmpC and MBLs are poorly inhibited by β-lactamase inhibitors and are active against cephamycins (4, 6, 11). AmpC enzymes are usually less active against cefepime and cefpirome than ESBLs and MBLs (11), and the activity of MBLs can be blocked by chelating agents (6). On the basis of the characteristics of these β-lactamases, various phenotypic detection methods have been proposed previously (1, 3, 5, 7, 11, 14, 20); however, coexistence of different classes of β-lactamases in a single bacterial isolate may pose diagnostic challenges.

We have detected AmpC enzymes and MBLs in cephamycin- and extended-spectrum cephalosporin-resistant *K. pneumoniae* (CECR-KP) isolates in Taiwan (16–19). Recently, the number of CECR-KP isolates that had resistance phenotypes that were slightly different than those previously reported for our AmpC and MBL producers seemed to be on the increase in our laboratory. These isolates were characterized in the present study, and the complexity of CECR-KP isolates was determined.

After excluding the previously reported isolates (17–19), a total of 99 nonreplicate CECR-KP isolates consecutively collected between January 1999 and June 2002 at the National Cheng Kung University Hospital, a 900-bed teaching hospital, were analyzed. These isolates were considered possible ESBL producers in accordance with the results of initial screening tests proposed by the National Committee for Clinical Laboratory Standards (NCCLS) and demonstrated resistance to cefoxitin in the standard disk diffusion tests (8).

TABLE 1. Categorization of CECR-KP isolates according to the results of phenotypic detection methods for AmpC, ESBLs, and MBLs

Isolate group	No. of isolates	Three-dimensional test result for AmpC detection[a]	Double-disk test result for ESBL detection	Double-disk test result for MBL detection	NCCLS confirmatory test result for ESBLs		
					Zone diameter change on CAZ (mm)[b]	Zone diameter change on CTX (mm)[b]	No. (%) of test-positive isolates
1a	30	Positive	Negative	Negative	+1 to +4	0 to +1	0 (0)
1b	12	Positive	Negative	Negative	−4 to −8	0 to −5	0 (0)
2a	10	Positive	Positive	Negative	−2 to −4	+8 to +14	9 (100)
2b	6	Positive	Positive	Negative	+1 to +3	+12 to +14	5 (100)
2c	19	Positive	Positive	Negative	+1 to +5	+2 to +6	9 (47.4)
3	2	Positive	Negative	Positive	+4, +6	+2, +4	1 (50.0)
4	9	Negative or indeterminate	Positive	Negative	+6 to +17	+7 to +14	9 (100)
5	11	Negative or indeterminate	Negative	Negative	+1 to +3	+1 to +3	11 (0)

[a] The results were interpreted and grouped as described by Manchanda and Singh (7).
[b] Changes in zone diameters of disks of ceftazidime (CAZ) and cefotaxime (CTX) plus clavulanic acid in comparison with those of drug disks-without clavulanic acid.

* Corresponding author. Mailing address: Department of Medical Technology, College of Medicine, National Cheng Kung University, No. 1 University Rd., Tainan, Taiwan 70101. Phone: 886-6-2353535 ext. 5775. Fax: 886-6-2363956. E-mail: jjwu@mail.ncku.edu.tw.

TABLE 2. Results of isoelectric focusing, β-lactamase characterization, susceptibility testing, and pulsed-field gel electrophoresis analysis

Isolate group	pI(s)	β-Lactamase(s)[a]	No. of isolates	Range of MICs (μg/ml)[b]									PFGE pattern(s) (no. of isolates)[c]
				AMC	FOX	CAZ	CAZ + CLA	CTX	CTX + CLA	ATM	FEP	IPM	
1a	9.0, 7.6	CMY-2, NE-SHV	7	64–128	128–256	64–>256	64–>256	16–64	16–32	16–32	0.13–0.5	0.25–1	
	9.0, 7.6, 5.4	CMY-2, NE-SHV, TEM-1	23	64–128 (64/128)	128–>256 (128/>256)	64–>256 (128/>256)	64–>256 (128/>256)	16–64 (32/64)	8–32 (16/32)	16–64 (16/32)	0.13–0.5 (0.25/0.5)	0.25–8 (0.5/1)	
1b	7.8, 7.6, 5.4	DHA-1, NE-SHV, TEM-1	12	64–128 (64/128)	128–>256 (256/>256)	16–32 (16/32)	64–256 (64/256)	2–16 (4/16)	4–64 (16/64)	2–16 (4/16)	0.06–0.25 (0.13/0.25)	0.5–1 (0.5/1)	Ia (3), XI (1), XIII (1), XV (1), XV (1), XVI (1), XVIII (1)
2a	8.4, 7.8, 7.6, 5.4	CTX-M-3, DHA-1, NE-SHV, TEM-1	9	64–128	≥128	32	64–128	32–64	2–16	16–64	8–32	0.5–2	Ic (1), II (1), III (1), VIII (1), XVII (1)
	7.9, 7.8, 7.6, 5.4	CTX-M-14, DHA-1, NE-SHV, TEM-1	1	64	>256	16	32	128	16	16	32	2	VI (1)
2b	8.4, 8.2, 7.8, 7.6, 5.4	CTX-M-3, E-SHV, DHA-1, NE-SHV, TEM-1	5	64–128	≥256	≥256	64–128	32–128	4–16	≥256	8–16	0.5–2	Id (1), X (1), XIV (1)
	8.2, 7.9, 7.8, 7.6, 5.4	E-SHV, CTX-M-14, DHA-1, NE-SHV, TEM-1	1	64	256	>256	128	128	8	>256	64	1	
2c	8.2, 7.8, 7.6, 5.4	E-SHV, DHA-1, NE-SHV, TEM-1	8	64–128	≥128	≥128	64–128	8–32	4–16	≥128	2–8	0.5–2	Ib (2), IV (1), V (1), VII (1), IX (1), XII (1)
	8.4, 8.25, 7.6, 5.4	CTX-M-3, CMY-8, NE-SHV, TEM-1	9	16–32	>256	16–64	8–32	64–256	16–64	8–64	2–16	0.25–1	
	9.0, 8.4, 7.6, 5.4	CMY-2, CTX-M-3, NE-SHV, TEM-1	1	64	>256	256	128	256	128	256	4	2	
	9.0, 8.4, 8.2, 7.6, 5.4	CMY-2, CTX-M-3, E-SHV, NE-SHV, TEM-1	1	128	>256	>256	128	>256	128	64	256	4	
3	8.2, 7.6, 5.4	IMP-8, NE-SHV, TEM-1	1	64	>256	>256	256	32	32	64	8	1	
	8.2, 7.6, 5.4	IMP-8, E-SHV, NE-SHV, TEM-1	1	64	>256	>256	256	32	32	0.25	4	2	
4	8.2, 7.6, 5.4	E-SHV, NE-SHV, TEM-1	3	8–16	32–64	≥256	8–16	64–256	16–32	≥256	8–16	0.5–1	
	8.2, 7.6	E-SHV, NE-SHV	2	16, 32	32, 64	>256	16	128, 256	8	>256	8, 16	1	
	7.9, 7.6, 5.4	CTX-M-14, NE-SHV, TEM-1	1	64	32	16	8	128	8	32	8	1	
	7.9, 7.6	CTX-M-14, NE-SHV	2	64	32	16, 32	8, 16	64	2, 4	8, 16	4, 8	0.5, 1	
	7.6	E-SHV	1	32	32	64	16	128	8	64	2	1	
5	7.6, 5.4	NE-SHV, TEM-1	7	16–64	32–64	2–16	1–16	2–16	1–8	4–16	0.03–0.13	0.06–0.5	
	7.6	NE-SHV	4	16–64	32–64	1–16	0.5–16	2–16	2–16	2–16	0.03–0.13	0.06–0.5	

[a] E-SHV, SHV-type ESBL; NE-SHV, SHV-type non-ESBL.

[b] AMC, amoxicillin and clavulanic acid; FOX, cefoxitin; CAZ, ceftazidime; CAZ + CLA, ceftazidime and clavulanic acid (2 μg/ml); CTX, cefotaxime; CTX + CLA, cefotaxime and clavulanic acid (2 μg/ml); ATM, aztreonam; FEP, cefepime; IPM, imipenem. The MIC values at which 50% of the isolates tested were inhibited (MIC_{50}) and MIC_{90} values are shown in parentheses (MIC_{50}/MIC_{90}).

[c] Only 24 randomly selected DHA-1-producing isolates were investigated. PFGE, pulsed-field gel electrophoresis.

The disk diffusion confirmatory tests for the presence of ESBLs were performed in accordance with NCCLS guidelines (8). The double-disk synergy test was also performed by placing a disk of cefepime (30 μg) at a distance of 20 mm (center to center) from a disk containing amoxicillin and clavulanic acid (20 and 10 μg) (14). The method has been reported to be sensitive in the detection of ESBLs in enterobacteria that have intrinsic AmpC enzymes (14). The three-dimensional extraction method proposed by Coudron et al. (5) was used with cefoxitin disks to detect AmpC-producing isolates. A modified Arakawa's double-disk test for detecting MBLs was performed by placing disks of ceftazidime and cefepime (30 μg each) with and without clavulanic acid (10 μg) at a distance of 25 mm (center to center) from a disk containing 2-mercaptopropionic acid (Sigma Chemical Co., St. Louis, Mo.) (1, 20). An enhanced zone of inhibition between the 2-mercaptopropionic acid disk and any one of the four drug disks was interpreted as a positive test result (20). The 99 CECR-KP isolates were divided into five major groups according to the results of the screening tests for AmpC and MBLs and the double-disk tests with cefepime for ESBLs (Table 1). Group 1 and 2 isolates were further subgrouped according to the results of the NCCLS confirmatory tests for the presence of ESBLs.

The expression of β-lactamases was detected by isoelectric focusing and the enzyme inhibition assay as described previously (16). PCR detection of bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M\text{-}1}}$-related, $bla_{\mathrm{CTX\text{-}M\text{-}9}}$-related, $bla_{\mathrm{CMY\text{-}8}}$, $bla_{\mathrm{CMY\text{-}2}}$, $bla_{\mathrm{DHA\text{-}1}}$-related, and $bla_{\mathrm{IMP\text{-}8}}$ genes was performed with previously reported oligonucleotide primers (10, 12, 15–18). The PCR-NheI method was used to discriminate between $bla_{\mathrm{SHV\text{-}ESBL}}$ and $bla_{\mathrm{SHV\text{-}non\text{-}ESBL}}$ genes (10). Amplification products of the other genes obtained from two independent PCRs were purified and sequenced twice. pIs of β-lactamases and the corresponding β-lactamase types determined by PCR assays are shown in Table 2. In the PCR-NheI test, coexistence of an undigested band and digested bands on an agarose gel for a single isolate with pI 7.6 and 8.2 β-lactamases suggested coproduction of an SHV-1-related non-ESBL and an SHV-5-related ESBL (4). MICs of various antimicrobial agents for the 99 CECR-KP isolates were determined by the standard agar dilution method with *Escherichia coli* ATCC 25922 as the control strain (9). The antimicrobial agents tested and the results are shown in Table 2.

Group 1 isolates were positive for the AmpC screening tests only. Subgroup 1b isolates demonstrated reduced zone diameters for ceftazidime and cefotaxime with clavulanic acid versus those for ceftazidime and cefotaxime tested alone in the ESBL confirmatory tests, and they were all found to be DHA-1 producers. These data are consistent with the inducibility of $bla_{\mathrm{DHA\text{-}1}}$ (2, 18). Subgroup 1a isolates were all CMY-2 producers.

Coproduction of AmpC and ESBLs in group 2 isolates was suggested by the positive results of the AmpC screening tests and the double-disk tests for ESBLs. DHA-1 and CTX-M-type ESBLs were detected in all subgroup 2a and 2b isolates; moreover, SHV-type ESBLs were detected in subgroup 2b isolates. The presence of CTX-M-type ESBLs might be responsible for markedly increased zone diameters of cefotaxime disks plus clavulanic acid in the ESBL confirmatory tests; the presence of SHV-type ESBLs in subgroup 2b isolates might mask the effect of DHA-1 on ceftazidime disks. The changes in the zone diameters of drug disks in the ESBL confirmatory tests were less evident for subgroup 2c isolates, and only 9 of 19 (47.4%) subgroup 2c isolates could be classified as ESBL producers by the ESBL confirmatory tests. Subgroup 2c isolates were found to coproduce an AmpC enzyme (DHA-1, CMY-2, or CMY-8) and one or two ESBLs (CTX-M and/or SHV-type ESBLs). The phenotypic characteristics of subgroup 2c and 1a isolates were similar except that group 2c isolates gave positive results with the double-disk tests with cefepime and demonstrated reduced susceptibilities to cefepime (Table 2).

Group 3 included two IMP-8-type MBL producers. The false-positive results given by the two isolates in the AmpC screening tests should be due to the hydrolysis of cefoxitin by IMP-8. One of these isolates also produced an SHV-type ESBL and showed a much higher aztreonam MIC than the other isolate that had no ESBL (64 versus 0.25 μg/ml).

No AmpC enzymes were detected by isoelectric focusing and PCR assays in group 4 and 5 isolates. Production of ESBLs was inferred by the phenotypic detection methods, and CTX-M or SHV ESBLs were detected in group 4 isolates. Reduced susceptibilities to cefoxitin in group 4 and 5 isolates could be due to mechanisms other than production of β-lactamases.

Pulsed-field gel electrophoresis of DNA samples from 24 isolates randomly selected from the DHA-1-producing isolates was performed after cleavage with the restriction endonuclease XbaI (New England Biolabs, Beverly, Mass.) (13, 16), and the results were interpreted according to Tenover's criteria (13). A total of 18 major patterns were obtained among the 24 isolates (Table 2). Four isolates coproducing DHA-1 and ESBLs and three isolates producing DHA-1 alone had similar patterns (patterns Ia to Id). Different patterns were also obtained among isolates with the same β-lactamase contents.

In conclusion, the present study demonstrated the complexity of CECR-KP isolates at a Taiwanese university hospital. The molecular typing analyses suggest that the complexity of these isolates could be due to stepwise acquisition of resistance determinants and acquisition of the same resistance determinants by different clones. Diagnostic problems posed by coexistence of different classes of β-lactamases in a single bacterial isolate could be solved by the combined use of various phenotypic detection methods. For epidemiologic purposes, the combined use of these phenotypic methods may be needed for microbiology laboratories in which high rates of CECR-KP isolates produce multiple β-lactamases.

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