JOURNAL OF CLINICAL MICROBIOLOGY, May 1998, p. 1446–1449 Vol. 36, No. 5
0095-1137/98/$04.00+0
Copyright © 1998, American Society for Microbiology

Survey of *Klebsiella pneumoniae* Strains Producing Extended-Spectrum β-Lactamases: Prevalence of SHV-12 and SHV-2a in Korea

JUNGMIN KIM,[1]* YOUNGMI KWON,[1] HYUNJOO PAI,[2] JONG-WON KIM,[3] AND DONG-TAEK CHO[4]

Department of Microbiology[1] and Department of Internal Medicine,[2] College of Medicine, Dankook University, Chonan, Department of Clinical Pathology, College of Medicine, Sungkyunkwan University, Seoul,[3] and Department of Microbiology, College of Medicine, Kyungpook University, Taegu,[4] Korea

Received 23 October 1997/Returned for modification 20 January 1998/Accepted 20 February 1998

Fifty-three clinical isolates of extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* collected from three hospitals in Korea were investigated for phenotypical and genotypical characterizations. Among these, 39 strains (74%) were shown by isoelectric focusing to carry SHV-type β-lactamases: 27 strains showed the pI 8.2 β-lactamase, and another 12 strains showed the pI 7.6 β-lactamase. The SHV gene of each of these strains was amplified by PCR, followed by nucleotide sequencing analysis. The gene of the pI 8.2 β-lactamase was found to be identical to the sequences encoding SHV-12, and the gene of the pI 7.6 β-lactamase was identical to the sequences encoding SHV-2a. A total of eight cefoxitin-resistant strains were found to have the plasmid-mediated AmpC-type β-lactamase, with a pI of 8.0, and this was confirmed to be CMY-1 β-lactamase by PCR and hybridization analysis. Noteworthy in this study is the fact that SHV-12 and SHV-2a have been the most commonly identified SHV-type ESBLs in Korea.

Among gram-negative pathogens in Korea, the incidence of resistance to extended-spectrum β-lactam antibiotics is becoming an ever-increasing problem. Extended-spectrum β-lactamases (ESBLs), such as the plasmid-mediated class-A TEM- and SHV-type enzymes, have developed by stepwise mutations in their structural genes, resulting in either single or multiple amino acid changes in the encoded enzymes. They confer variable levels of resistance to cefotaxime, ceftazidime, and other broad-spectrum cephalosporins and to monobactams such as aztreonam, but they have no detectable activity against cephamycins and carbapenems (9, 17, 18). Recently, new plasmid-mediated ESBLs, not derived from TEM or SHV enzymes but related to cephalosporinases of *Enterobacteriaceae* (AmpC enzymes), that confer resistance to all cephalosporins, including cephamycins such as cefoxitin, have been reported (2, 3, 9, 18). While the frequency of ceftazidime-resistant *Klebsiella pneumoniae* has been high in Korea, no significant information on the ESBLs from Korean isolates has been available. We performed a study with clinical isolates of *K. pneumoniae* collected from three hospitals in Korea to investigate the ESBL-producing isolates for phenotypical and genotypical characteristics.

Bacterial strains. Fifty-three clinical isolates of ESBL-producing *K. pneumoniae* collected from three hospitals in Korea were used. Initially, 90 clinical isolates of *K. pneumoniae* were randomly collected from Dankook University Hospital and Seoul National University Hospital in 1996 and were tested for ESBL production by double-disk synergy test; 20 of the strains tested showed clavulanic acid enhancement of oxyimino-β-lactam susceptibilities. Additionally, 33 ESBL-producing strains confirmed by double-disk synergy test were donated from Yonsei University Hospital.

* Corresponding author. Mailing address: Department of Microbiology, College of Medicine, Dankook University, San 29, Anseo-dong, Chonan, Chungnam, 330-180, Korea. Phone: 82-417-550-3853. Fax: 82-417-550-3905. E-mail: minkim@ns.anseo.dankook.ac.kr.

Analytical isoelectric focusing. Fifty-three ESBL-producing *K. pneumoniae* isolates were subjected to isoelectric focusing. Crude β-lactamases were prepared by sonication, and isoelectric focusing of the enzyme preparations was performed in polyacrylamide gels with a pH range of 3.5 to 9.5 by a modified Matthew method (11). Among 53 ESBL-producing isolates of *K. pneumoniae*, 27 strains produced β-lactamase consistent with a pI of 8.2, 12 strains produced β-lactamase with a pI of 7.6, 13 strains produced TEM-type β-lactamase with pIs of 5.4 and 5.9, and 8 strains produced β-lactamase with a pI of 8.0. Seven strains produced multiple enzymes, as many as three per strain, with pIs of 5.4, 5.9, and 8.2 (two strains); 5.4, 5.9, and 8.0 (two strains); 8.0 and 8.2 (one strain); 7.6 and 8.0 (one strain); and 7.6 and 5.4 (one strain). The majority of the clinical isolates also produced chromosomally encoded β-lactamase of *K. pneumoniae*, as well as ESBLs.

Characterization of SHV ESBL-producing isolates. We characterized the SHV-type β-lactamases produced by 39 strains of *K. pneumoniae*. When the isolates were tested for the ability to transfer the bla_{SHV} gene, 10 of 12 isolates with the pI 7.6 β-lactamase transferred the bla_{SHV} gene by conjugation to *Escherichia coli* J53 Azi^r (5) via a large plasmid in the range of 30 to 106 kb, but the isolates with the pI 8.2 β-lactamase did not transfer the bla_{SHV} gene. The pI 8.2 β-lactamase-encoding plasmids were not self-transmissible, but the majority of these were mobilized by an R641 transfer factor. Selected transconjugants expressed the SHV-type β-lactamase alone or in combination with the pI 5.4 β-lactamase. MICs were determined in order to obtain a resistance pattern for each individual wild-type and transconjugant strain. The results are summarized in Table 1. For the strains expressing the pI 8.2 β-lactamase, the MICs at which 50% of the isolates were inhibited (MIC_{50}) of cefotaxime, ceftazidime, and aztreonam were 32, 256, and 512 μg/ml, respectively. For the strains expressing the pI 7.6 β-lactamase, the MICs of oxyimino-cephalosporins and aztreonam were relatively low. The presence of β-lactamase with a pI of 8.0 in two strains raised the MIC of cefoxitin to 64 and >512

TABLE 1. Antimicrobial susceptibilities and pIs of β-lactamases of *K. pneumoniae* clinical isolates and their transconjugants

pI(s) of β-lactamase(s)[b]	No. of strains	MIC_{50} (μg/ml) of[a]:					MIC_{90} (μg/ml) of:				
		AMP	CFX	CTX	CAZ	ATM	AMP	CFX	CTX	CAZ	ATM
Clinical isolates											
8.2	24	>512	8	32	256	512	>512	32	64	512	>512
7.6	10	>512	8	32	16	4	>512	16	64	64	128
8.2, 5.9[c]	1	>512	8	64	256	512					
8.2, 5.9[c]	1	>512	16	64	512	512					
8.2, 8.0[c]	1	>512	64	64	512	>512					
7.6, 8.0[c]	1	>512	>512	256	32	8					
7.6, 5.4[c]	1	>512	4	256	32	8					
Transconjugants											
8.2	19	>512	4	4	8	64	>512	8	16	32	256
7.6	9	512	4	2	<1	<1	>512	32	16	8	2
7.6, 5.4[c]	1	>512	4	16	8	2					
E. coli recipient[c]		4	4	0.25	0.5	0.125					

[a] MICs were determined in agar dilution tests (13). AMP, ampicillin; CFX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam.
[b] In addition to the chromosomal SHV-type β-lactamase of *K. pneumoniae.*
[c] MIC, not MIC_{50}.

μg/ml. On the basis of MIC pattern, we selected three strains of the pI 8.2 β-lactamase and eight strains of the pI 7.6 β-lactamase and performed PCR followed by nucleotide sequencing of the amplimers. An 870-bp fragment of the SHV gene was amplified with the primers S1 (5′-TGGTTATGCGTTATATT CGCC-3′) and S2 (5′-GGTTAGCGTTGCCAGTGCT-3′), corresponding to nucleotides 120 to 140 and 990 to 972, respectively, of the SHV-1 *bla* gene (12). DNA sequencing of the SHV gene was performed with amplimers on both strands with a dideoxy termination cycle sequencing kit (Perkin-Elmer Cetus, Norwalk, Conn.). The SHV-12-specific mutations (14) in the *bla* gene (glutamine 35, serine 238, and lysine 240; numbering according to the scheme of Ambler et al. [1]) were detected in all three strains of the pI 8.2 β-lactamase, and the SHV-2a-specific mutations (7, 16) in the *bla* gene (glutamine 35 and serine 238) were detected in all eight strains of the pI 7.6 β-lactamase. We performed a kinetic study with SHV-2, SHV-2a, and SHV-12. The relative hydrolysis rates of these enzymes for cephaloridine, cefotaxime, ceftazidime, and aztreonam are shown in Table 2. The substrate profiles of SHV-2a and SHV-12 were compared with that of SHV-2, the first described ESBL. The hydrolysis rates of SHV-2a for the tested substrates were similar to those of SHV-2. Compared with SHV-2a, SHV-12 showed an increasing hydrolysis rate for ceftazidime and, in particular, aztreonam. To determine on which plasmid the gene encoding the enzyme with a pI of 7.6 or the enzyme with a pI of 8.2 resided, Southern blotting was performed on clinical isolates by using the PCR product of the bla_{SHV} gene as a probe. The results indicated that the plasmid in the range of 64 to 121 kb carried an SHV-type β-lactamase.

TABLE 2. Substrate profiles of SHV-2, SHV-2a, and SHV-12 enzymes

β-Lactamase (pI)	Relative V_{max}[a]					Source or reference
	PCN	CER	CTX	CAZ	AZT	
SHV-2 (7.6)	100	123	27	<1	11	10
SHV-2a (7.6)	100	215	35	<1	11	This study
SHV-12 (8.2)	100	214	42	16	110	This study

[a] Hydrolytic activity against β-lactams was evaluated by spectrophotometry (model UVIKON 860; Kontron) at 37°C in 0.1 M phosphate buffer (pH 7.0). V_{max} was calculated by Lineweaver-Burk plot and expressed relative to the V_{max} for benzylpenicillin (taken as 100%). Abbreviations: PCN, benzylpenicillin; CER, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime; AZT, aztreonam.

Noteworthy in this study was the prevalence of SHV-12 (SHV-5-2a) and SHV-2a, because these enzymes have rarely been found in other countries. Furthermore, SHV-12 was very recently reported by Nüesch-Inderbinen et al. only in 2 isolates of 34 SHV-type ESBL-producing *Enterobacteriaceae* isolated from Swiss patients (14). The MICs of oxyimino-cephalosporins and aztreonam for the strains expressing SHV-2a β-lactamase were relatively low, while the strains expressing SHV-12 β-lactamase showed high resistance to oxyimino-cephalosporins and monobactams. Our kinetic study also demonstrated that SHV-12 hydrolyzed ceftazidime and aztreonam far more efficiently than did SHV-2a. Analysis of the results of nucleotide sequence determination showed that SHV-12 differs from SHV-2a by an amino acid change from glutamic acid to lysine at position 240. So this change of the gene coding for SHV-12 is thought to contribute to the expansion of the spectrum of this enzyme to include ceftazidime and aztreonam, as in the case of SHV-5. The substrate profile and the sites of amino acid variation suggested that SHV-12 was derived from SHV-2a, by replacement of glutamic acid with lysine at position 240. Continued challenge of an SHV-2a-producing strain with 7-oxyimino-cephalosporins, particularly ceftazidime, is likely to select for the SHV-12 mutation. In an isoelectric focusing test, crude extracts prepared from strains expressing the SHV-12 β-lactamase showed a band which aligned with SHV-5 at a pI of 8.2. Although SHV-2a and SHV-12 were indistinguishable from SHV-2 and SHV-5 (6), respectively, by isoelectric point and substrate profile, SHV-2a and SHV-12 share the same substitution of glutamine for leucine at position 35 compared with SHV-2 and SHV-5, respectively. This point mutation, though far from the active site and known not to alter the isoelectric point (16), could cast light on the evolutionary relationships among the SHV-type enzymes. It is now known that the genes encoding ESBLs have also acquired silent mutations which are useful in tracing their evolution. In a comparison of the nucleotide sequences, SHV-2a and SHV-12 genes in all tested strains shared the same silent mutations in the coding triplets for leucine 138 (CT**G**) and for threonine 268 (AC**G**) (letters in boldface represent mutated nucleotides). In con-

TABLE 3. Antimicrobial susceptibilities, pIs of β-lactamases, and plasmid sizes of CMY-1 producing *K. pneumoniae* strains

Strain[a]	MIC (μg/ml) of[b]:					pI(s) of β-lactamase(s)[c]	Plasmid size(s) (kb)
	AMP	CFX	CTX	CAZ	ATM		
KY02	>512	512	32	2	<1	8.0	130, 77
pKY2	128	64	16	<1	<1	8.0	77
KY03	>512	256	8	4	<1	8.0	130, 77
pKY3	128	64	16	<1	<1	8.0	77
KY04	128	64	8	4	<1	8.0	130, 77
KD08	>512	128	16	4	4	8.0	130
pKD8	128	256	32	2	2	8.0	130
KY23	>512	64	64	512	>512	8.0, 8.2	142, 106
KY29	>512	>512	256	32	8	8.0, 7.6	142, 106
KY05	>512	>512	>512	512	512	8.0, 5.4, 5.9	142, 83
K S18	>512	>512	128	>512	>512	8.0, 5.4, 5.9	142, 83

[a] pKY2, pKY3, and pKD8 are transconjugants.
[b] Abbreviations: AMP, ampicillin; CFX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam.
[c] In addition to the chromosomal SHV-type β-lactamase of *K. pneumoniae.*

trast, it has been reported that SHV-2 and SHV-5 (4, 7, 8, 15) contain leucine 138 encoded by CT**A** and threonine 268 encoded by AC**C**. These findings, together with the unusual prevalence of SHV-2a and SHV-12 in Korea, lead to two speculations: one is that SHV-2a and SHV-2 may have a separate evolutionary development; the other is that SHV-2a and SHV-12 may have evolved from a common ancestor in the sequential order of SHV-2a first, followed by SHV-12.

Characterization of the pI 8.0 β-lactamase. Among 53 ESBL-producing strains of *K. pneumoniae*, 8 strains produced the pI 8.0 β-lactamase either alone or in combination with another type of β-lactamase and were resistant to ampicillin, with MICs above 128 μg/ml, and to cefoxitin, with MICs ranging from 64 to >512 μg/ml (Table 3). Four strains that carried the pI 8.0 β-lactamase only revealed borderline susceptibility to cefotaxime, with MICs ranging from 8 to 32 μg/ml, and susceptibility to ceftazidime and aztreonam, with MICs of 4 μg/ml or lower. Three of eight strains transferred the *bla* gene in a conjugation experiment and possessed plasmids with molecular sizes of approximately 77 or 130 kb. The resulting transconjugants expressed the pI 8.0 β-lactamase and were resistant to cefoxitin. To determine whether this enzyme was an SHV-related ESBL or a plasmid-borne AmpC enzyme, we performed parallel tests in the presence and absence of 0.1 mM clavulanate and 0.1 mM cloxacillin. The pI 8.0 β-lactamase was inhibited by cloxacillin but not by clavulanic acid. Since the phenotype of the pI 8.0 β-lactamase was similar to that of CMY-1 (3), primers were selected from the CMY-1 sequence (2) to amplify the *bla* genes of the transconjugants and clinical isolates. The primers C1 (5′-ATGCAACAACGA CAATCC-ATC-3′) and C2 (5′-GTTGGGGTAGTTGC-GAT TGG-3′), corresponding to nucleotides 1 to 21 and 1098 to 1078, respectively, of the CMY-1 *bla* gene (5), were used. bla_{CMY-1} genes were amplified in all the strains expressing the pI 8.0 β-lactamase, and the result was confirmed by the hybridization of plasmid with a CMY-1 gene probe (data not shown). The finding that the MICs of ceftazidime were lower than those of cefotaxime for strains producing CMY-1 is unusual because for most cephamycinase-producing strains, either the MICs of both compounds are equal or the MICs of ceftazidime are higher than those of cefotaxime (e.g., CMY-2, MIR-1, BIL-1, LAT-1, and FOX-1) (2, 3). In 1989 CMY-1 was identified as the first plasmid-mediated AmpC-type β-lactamase in *K. pneumoniae* CHO (3); it was found in an isolate from a patient in Yonsei University Hospital, which is one of the three hospitals involved in this study. While we studied a limited number of strains, CMY-1 has been prevalent in cefoxitin-resistant *K. pneumoniae* isolated in Korea since its discovery in 1989. Since the additional selective pressure imposed by use of cephamycins and β-lactamase inhibitors will favor strains producing AmpC-type β-lactamases, more prudent use of antibiotics is necessary to reduce the spread of these resistant strains.

This work was supported by the Mission Oriented Grant KOSEF 971-0712-102-2 from the Korean Science and Engineering Foundation.

REFERENCES

1. **Ambler, R. P., A. F. W. Coulson, J. M. Frere, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–270.
2. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, R. Wilhelm, and Y. Chong.** 1996. Comparative characterization of the cephamycinase bla_{CMY-1} gene and its relationship with other β-lactamase genes. Antimicrob. Agents Chemother. **40:**1926–1930.
3. **Bauernfeind, A., Y. Chong, and S. Schweighart.** 1989. Extended broad spectrum β-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. Infection **17:**316–321.
4. **Billot-Klein, D., L. Gutmann, and E. Collatz.** 1990. Nucleotide sequence of the SHV-5 β-lactamase gene of a *Klebsiella pneumoniae* plasmid. Antimicrob. Agents Chemother. **34:**2439–2441.
5. **Coetzee, J. N., N. Datta, and R. W. Hedges.** 1972. R factors from *Proteus rettgeri*. J. Gen. Microbiol. **72:**543–552.
6. **Du Bois, S. K., M. S. Marriott, and S. G. B. Amyes.** 1995. TEM- and SHV-derived extended-spectrum β-lactamases: relationship between selection, structure and function. J. Antimicrob. Chemother. **35:**7–22.
7. **Garbarg-Chenon, A., V. Godard, R. Labia, and J.-C. Nicolas.** 1990. Nucleotide sequence of SHV-2 β-lactamase gene. Antimicrob. Agents Chemother. **34:**1444–1446.
8. **Huletsky, A., F. Couture, and R. C. Levesque.** 1990. Nucleotide sequence and phylogeny of SHV-2 β-lactamase. Antimicrob. Agents Chemother. **34:**1725–1732.
9. **Jacoby, G. A., and A. A. Medeiros.** 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **35:**1697–1704.
10. **Jacoby, G. A., and L. Sutton.** 1991. Properties of plasmids responsible for

extended-spectrum β-lactamase production. Antimicrob. Agents Chemother. **35:**164–169.
11. **Matthew, M. A., A. M. Harris, M. J. Marshall, and G. W. Rose.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. **88:**169–178.
12. **Mercier, J., and R. C. Levesque.** 1990. Cloning of SHV-2, OHIO-1, and OXA-6 β-lactamases and cloning and sequencing of SHV-1 β-lactamase. Antimicrob. Agents Chemother. **34:**1577–1583.
13. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 7th ed. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Villanova, Pa.
14. **Nüesch-Inderbinen, M. T., F. H. Kayser, and H. Hächler.** 1997. Survey and molecular genetics of SHV β-lactamases in *Enterobacteriaceae* in Switzerland: two novel enzymes, SHV-11 and SHV-12. Antimicrob. Agents Chemother. **41:**943–949.
15. **Podbielski, A., and B. Melzer.** 1990. Nucleotide sequence of the gene encoding the SHV-2 β-lactamases (bla_{SHV-2}) of *Klebsiella ozaenae*. Nucleic Acids Res. **18:**4916.
16. **Podbielski, A., J. Schonling, B. Melzer, K. Warnatz, and H.-G. Leusch.** 1991. Molecular characterization of a new plasmid-encoded SHV-type β-lactamase (SHV-2 variant) conferring high-level cefotaxime resistance upon *Klebsiella pneumoniae*. J. Gen. Microbiol. **137:**569–578.
17. **Sanders, C. C., and W. E. Sanders.** 1992. β-Lactam resistance in gram-negative bacteria: global trends and clinical impact. Clin. Infect. Dis. **15:**824–839.
18. **Sirot, D.** 1995. Extended-spectrum plasmid-mediated β-lactamases. J. Antimicrob. Chemother. **36**(Suppl. A)**:**19–34.