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Emergence and Wide Dissemination of CTX-M-type ESBLs, and CMY-2- and DHA-1-type AmpC β-Lactamases in Korean Respiratory Isolates of *Klebsiella pneumoniae*

Respiratory isolates of *Klebsiella pneumoniae* in Korea during 2002-2003 were studied to determine the prevalence and types of extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases (PABLs). ESBL-production was tested by double-disk synergy, and genotypes of β-lactamases were determined by PCR and sequencing. ESBLs were detected in 28.4% of 373 isolates, and the most prevalent types were SHV-12 (63 isolates) and CTX-M-14 (9 isolates). Forty of 75 ESBL-producers (53.5%) also had PABLs: 21 isolates with CMY-2-like, 17 with DHA-1-like. Pulsed-field gel electrophoresis showed 19 types and 25 of 74 isolates had an identical pattern, indicating nosocomial spread. Dissemination of ESBL- and PABL-producing *K. pneumoniae* strains in Korea is a particular concern, as it limits the choice of antimicrobial agents for treatment of infections.

Key Words : *beta-lactamase CTX-M-14; beta-lactamase CMY-2; beta-lactamase DHA-1; AmpC beta-lactamase; Klebsiella pneumoniae*

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INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) produced by Gram-negative bacilli have been a serious problem in hospitalized patients worldwide (1). These enzymes are most commonly produced by strains of *Klebsiella pneumoniae*, one of the important nosocomial respiratory pathogens. ESBLs have been very prevalent in Korea most being of the types, SHV-12, SHV-2a, and TEM-52, however, CTX-M types have emerged recently as well (2, 3). ESBLs do not hydrolyze cephamycins, however, the cephamycin-hydrolyzing plasmid-mediated AmpC β-lactamase (PABL), CMY-1, emerged in Korea in 1988 (4). As well, various types of PABLs have been increasingly detected in intrinsically cephamycin-susceptible Gram-negative bacilli in other parts of the world (5, 6). In 2003, 36% and 37% of *K. pneumoniae* isolates at a Korean tertiary-care hospital showed resistance to ceftazidime and cefoxitin, respectively (unpublished data). These results suggest a high prevalence of ESBL and PABL-producing isolates. Recent increases of *K. pneumoniae* isolates concomitantly producing PABL and ESBL pose a serious therapeutic problem as carbapenems remain the only active β-lactams against these organisms (7).

This study was conducted to determine the prevalence and the types of ESBLs and PABLs among *K. pneumoniae* isolates from respiratory specimens. This information is crucial for controlling of the spread of resistance and for the decision of empirical selection of antimicrobial agents.

MATERIALS AND METHODS

Clinical isolates and antimicrobial susceptibility testing

A total of 373 non-duplicate isolates of *K. pneumoniae*, excluding scant growth, were isolated from the lower respiratory specimens of patients in a Korean tertiary-care hospital in Seoul over a one year period starting in September 2002. The isolates were identified by the conventional methods (8) or by using the Vitek GNI card (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility was tested by the National Committee for Clinical Laboratory Standards (NCCLS) disk diffusion method (9). ESBL production was detected with the

double-disk synergy test with cefotaxime, ceftazidime, aztreonam, cefepime, and amoxicillin/clavulanic acid disks, or phenotypic confirmatory tests as recommended by the NCCLS (9).

MICs of β-lactams were determined by the NCCLS agar dilution method (9). Antimicrobial agents used were cephalothin and cefepime (Sigma, St. Louis, MO, U.S.A.), ceftazidime and clavulanic acid (GlaxoSmithKline, Greenford, U.K.), cefotaxime (Aventis, Frankfurt, Germany), cefoxitin and imipenem (Merck/Sharp & Dohme, Rahway, NJ, U.S.A.), and aztreonam (Bristol-Myers Squibb, Princeton, NJ, U.S.A.).

Isoelectric focusing and resistance transfer

Isoelectric focusing was carried out following the manufacturer's instructions using pre-cast gels with pH gradient 3-10, and a ThermoFlow Electrophoresis Temperature Control System (Novex Experimental Technologies, San Diego, CA, U.S.A.). Resistance transfer was tested by an agar mating method with nalidixic acid-resistant recipient *E. coli* RG 176, rifampicin-resistant *E. coli* RG 488, or azide-resistant *E. coli* J53. Mueller-Hinton agar containing 100 mg/L of nalidixic acid, rifampicin or azide, and 2 mg/L of ceftriaxone or aztreonam was used to select transconjugants.

Molecular methods

PCR for the detection of *bla*$_{TEM}$ and *bla*$_{SHV}$ in the transconjugants was carried out as described previously (10). PCR for the detection of *bla*$_{CTX-M}$ in ESBL-producing clinical isolates was performed with primers CTXUNI-F (5′-CVATGTGCAGYACCAGTAA-3′) and CTXUNI-R (5′-ARGTSACCAGAAYMAGCGG-3′). A thermocycler (Eppendorf, Hamburg, Germany) was used under the following conditions: 94℃ for 5 min, 35 cycles of 94℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 45 sec, followed by 72℃ for 7 min. PCR for detection of family-specific PABL gene alleles in cefoxitin-intermediate or -resistant isolates was performed by the method of Perez-Perez and Hanson with slight modification (11). PCR for detection of *bla*$_{DHA}$ in DHA PCR positive isolates was performed using the forward primer DHA1-F (5′-TCTGCCGCTGATAATG TCG-3′) for detection of *bla*$_{DHA-1}$ and *bla*$_{DHA-2}$; reverse primers were DHA1-R (5′-GCCGCCGGATCATTCAGCGC-3′) and DHA2-R (5′-TCTGCCGGGTCATTCAACAT-3′), respectively.

Sequencing was performed using the following primers: for *bla*$_{CTX-M}$, CTXM-F (5′-AAAAATGATTGAAAGGTGGTTGT-3′) and CTXM-R (5′-TTACAGCCCTTCGGCGATGA-3′); for *bla*$_{CMY-1}$, CMY1E-F (5′-TATTAGAGCGGTTTAGGCTG-3′) and CMY1E-R (5′-AATGTACCGCCCTCTTTC-3′); for *bla*$_{CMY-2}$, CITS-F (5′-AACACACTGATTGCGTCTGA-3′) and CITS-R (5′-TCCTGGGCCTCATCGTCAGTTAT-3′). Sequencing of *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{DHA}$ was performed with the primers as described previously (10, 11). DNAs in the PCR products were extracted with a gel extraction kit (Qiagen, Hilden, Germany) and used for direct sequencing of ESBL and PABL genes. Both strands were analyzed by the dideoxy-chain termination method with an ABI 3700 Autosequencer (Perkin-Elmer, Foster City, CA, U.S.A.).

Genomic DNA from *K. pneumoniae* isolates was digested using *Xba*I (Takara, Japan) and separated using a CHEF-DRII system according to the manufacturer's instruction (Bio-Rad, Hercules, CA, U.S.A.). The pulsed-field gel electrophoresis (PFGE) band patterns were analyzed with the computer software UVIBand Map V.99 (UVItec Ltd., Cambridge, U.K.) by the un-weighted pair group method using arithmetic averages (UPGMA).

RESULTS

Among the 373 *K. pneumoniae* isolates, 106 (28.4%) tested positive using either the double-disk synergy test or NCCLS confirmatory test, indicating ESBL production. Resistance was transferable in 75 (70.8%) of the isolates. Seventy-six ESBL genes were detected by PCR in 75 transconjugants (Table 1).

The most common ESBL type, SHV-12, was identified in 26 of 63 *bla*$_{SHV}$ allele-positive isolates, which showed β-lactamase bands with a pI of 8.2. The second most common type was CTX-M-14, being detected in nine isolates. SHV-2a and TEM-52 were identified in only one and two isolates, respectively.

Forty of 75 (53.3%) clinical isolates with ESBL genes had PABLs. Twenty one contained *bla*$_{CMY-2}$-like, 17 contained *bla*$_{DHA-1}$-like, and two contained *bla*$_{CMY-1}$ (Table 1). Antibiotic resistance by disk diffusion method of isolates without ESBL and PABL genes, those with ESBL genes, and those with both ESBL and PABL genes were: to cefoxitin 6%, 3%, and 100%; to aminoglycosides 5-9%, 40-97%, and 90-100%; and to levofloxacin 4%, 60%, and 80%, respectively.

The MIC$_{90}$s of β-lactams to ESBL-producers were: cefotaxime and ceftazidime for both PABL-nonproducer and -producers 64 mg/L and >128 mg/L, respectively; cefotaxime/clavu-

Table 1. Plasmid-mediated AmpC-β-lactamases detected from extended-spectrum β-lactamase-producing *K. pneumoniae* isolates

ESBL type (No. of isolates)	No. of isolates with AmpC β-lactamase			
	None	CMY-1	CMY-2	DHA-1
SHV-12 (26)	13	1 (1)*	3 (2)	9 (0)
SHV-12-like (37)	15	1 (1)	16 (2)	5 (2)
SHV-2a (1)	1	0	0	0
TEM-52 (2)	2	0	0	0
CTX-M-14 (8)	3	0	2 (0)	3 (0)
CTX-M-14+SHV-12 (1)	1	0	0	0
Total (75)	35	2 (2)	21 (4)	17 (2)

*The number of isolates with their AmpC genes sequenced is shown in parenthesis.

Table 2. Antimicrobial susceptibilities of ESBL-producing *K. pneumoniae* isolates with and without concomitant production of PABLs

ESBL-producing strains (No. of isolates)	Antimicrobial agent	MIC (mg/L)			Susceptibility (%)		
		Range	50%	90%	Susceptible	Intermechiate	Resistant
PABL-production positive (40)	Cephalothin	>128	>128	>128	0	0	100
	Ceftazidime	4->128	>128	>128	5.0	0	95.0
	Ceftazidime/clavulanate	0.5->128	128	128	-	-	-
	Cefotaxime	4->128	16	64	12.5	67.5	20.0
	Cefotaxime/clavulanate	0.12-64	16	32	-	-	-
	Cefoxitin	16->128	>128	>128	0	7.5	92.5
	Cefepime	0.5-128	2	8	92.5	0	7.5
	Aztreonam	16-128	64	64	0	2.5	97.5
	Imipenem	0.12-8	0.25	0.5	97.5	2.5	0
PABL-production negative (35)	Cephalothin	>128	>128	>128	0	0	100
	Ceftazidime	4->128	128	>128	5.7	0	96.3
	Ceftazidime/clavulanate	0.5-2	1	2	-	-	-
	Cefotaxime	8->128	16	64	17.1	62.9	20.0
	Cefotaxime/clavulanate	0.03-0.5	0.12	0.25	-	-	-
	Cefoxitin	2-16	8	16	71.4	28.6	0
	Cefepime	1-32	4	16	88.6	2.9	8.6
	Aztreonam	8-128	64	128	2.9	14.2	82.9
	Imipenem	0.12-1	0.12	0.5	100	0	0

lanate and ceftazidime/clavulanate for PABL-nonproducer 2 mg/L and 0.25 mg/L, respectively, and PABL-producer 32 mg/L and 128 mg/L, respectively; cefoxitin for PABL-nonproducer and -producer 16 mg/L and >128 mg/L, respectively (Table 2).

PFGE of *Xba*I-digested genomic DNA of 74 ESBL producers showed 19 types including nine subtypes. Twenty-five isolates gave an identical pattern, O1 (Fig. 1), which was mostly isolated from NCU patients. Among these, 18 isolates produced both SHV-12-like and CMY-2-like enzymes.

DISCUSSION

The prevalence of ESBLs among clinical isolates varies depending on countries and institutions. In the United States occurrence of ESBL production in Enterobacteriaceae ranges from 0 to 25%, with the national average being around 3% (1). ESBL production among *K. pneumoniae* isolates was comparatively higher in Taiwan and Hong Kong, 8.5% and 13%, respectively, but very low in Japan, less than 1% (1). The prevalence in our study during 2002-2003 was 28.4%, which is significantly higher than the United States, Taiwan, or Hong Kong, but still similar to that of other Korean study (10).

SHV-12, TEM-52, and SHV-2a were common in the late 1990s in Korea when compared to other countries (2). In this study, SHV-12-like enzyme remained prevalent (64 of 75), however SHV-2a and TEM-52 were found in only one and two isolates, respectively.

Recently, strains with CTX-M type ESBLs have been increasingly isolated from many parts of the world (1). In Korea, a few strains harboring CTX-M-14 β-lactamase have been reported since 2000 (3, 10). In this study, nine of 106 isolates harbored CTX-M-14 type ESBLs, indicating a gradual increase of this type.

Since 1989 over 20 types of PABLs have been reported worldwide (6). In 1995, CMY-1b was reported in Korea with a one amino acid change, Asn346Ile, compared to CMY-1 (5). It was later renamed CMY-10 (12). This type was however not detected in this study; neither was CMY-11 which was first identified in *E. coli* in 2002 in Korea (13).

Korean Antimicrobial Surveillances showed that the cefoxitin-resistance rate of *K. pneumoniae* increased from 14% in 1998 to 20% in 2001 (14), suggesting dissemination of PABL-producing isolates. In this study, 41 of 75 (54.7%) isolates were cefoxitin resistance, and 40 isolates had PABL genes. Isolates with $bla_{\text{CMY-2-like}}$ and $bla_{\text{DHA-1-like}}$ were detected in 21 and 17 isolates respectively, while $bla_{\text{CMY-1}}$ was found in only two isolates.

CMY-2 (BIL-1, LAT-2) was first identified in *K. pneumoniae* isolates from Greece in 1990 (15). CMY-2 was the most prevalent and most geographically distributed PABL, with reports from Africa, Europe, India, Taiwan, and the United States (6). It is a concern that as is ACT-1 enzyme, CMY-2 together with reduced permeability can render the isolate resistant even to imipenem (16, 17).

DHA-1, an inducible PABL, which was first identified in *Salmonella enteritidis* isolated in Saudi Arabia in 1992, has been reported in several other countries including Korea (18, 19). DHA-1-producing *K. pneumoniae* isolates were reported to be especially prevalent in a Korean university hospital (20).

ESBL or PABL-producing strains were often resistant to aminoglycosides and fluoroquinolones, too. In our study, isolates with both ESBLs and PABLs, were more often resistant to cefoxitin, aminoglycosides, and levofloxacin.

The MIC_{90}s of cefotaxime and ceftazidime for ESBL-producing strains were similar to those for strains producing both ESBLs and PABLs. Addition of clavulanic acid cefotaxime and

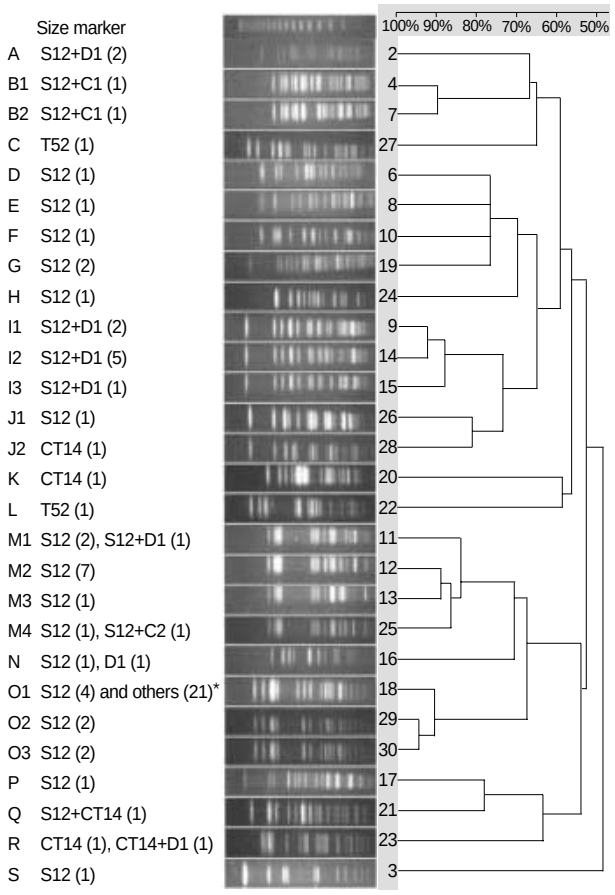

Fig. 1. PFGE band pattern of 74 strains of *K. pneumoniae* were analyzed with UVIBand Map V.99 by the un-weighted pair group method using arithmetic averages (UPGMA). *The β-lactamases produced include S12+C2 (18), S12+D1 (1), and CT14+C2 (2). C1, CMY-1; C2, CMY-2; CT14, CTX-M-14; D1, DHA-1; S12, SHV-12; T52, TEM-52.

ceftazidime reduced the MIC_{90}s for ESBL producers, but not for strains producing both ESBLs and PABLs.

In this study, PFGE of *Xba*I-digested genomic DNA showed that many isolates had the patterns O (29 isolates) and M (13 isolates). Some isolates with different ESBLs, or both ESBLs and PABLs, showed a few identical patterns, implying that both clonal and horizontal spread, and the propensity of *K. pneumoniae* to acquire resistance genes, has a role for their high prevalence in this study.

In conclusion, SHV-12 type ESBL-producing *K. pneumoniae* are increasing among lower respiratory isolates. As well, it seems that CTX-M-14-producing isolates are emerging in Korea. It also seems that the coexistence of PABLs such as CMY-2 and DHA-1 is common. Spreading of ESBL-producing strains with PABL genes is a concern, as it causes limitations in the selection of antimicrobial agents for optimal treatment of patients.

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