

# Prevalence of Plasmid-Mediated AmpC $\beta$ -Lactamases in a Chinese University Hospital from 2003 to 2005: First Report of CMY-2-Type AmpC $\beta$ -Lactamase Resistance in China<sup>∇</sup>

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The aim of this study was to investigate the prevalences of plasmid-mediated AmpC  $\beta$ -lactamases (PABLs) in isolates of *Escherichia coli* and *Klebsiella* spp. from a university hospital in China. A total of 1,935 consecutive nonrepeat clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* were collected between January 2003 and July 2005. The isolates with cefoxitin zone diameters less than 18 mm (screen positive) were selected for PCR of the *bla*<sub>AmpC</sub> genes and sequencing. Fifty-four (2.79%) isolates harbored PABLs, as demonstrated by PCR and isoelectric focusing. Sequence analysis revealed the presence of *bla*<sub>DHA-1</sub> and *bla*<sub>CMY-2</sub> genes. The Southern blot hybridization studies confirmed that *bla*<sub>CMY-2</sub> and *bla*<sub>DHA-1</sub> were located on plasmids. Based on species, PABLs were detected in 4.29% (29 isolates of DHA-1 and 1 isolate of CMY-2) of *K. pneumoniae*, 1.91% (11 isolates of DHA-1 and 12 isolates of CMY-2) of *E. coli*, and 3.03% (1 isolate of DHA-1) of *K. oxytoca* isolates. In contrast to our anticipation, the occurrence rate of DHA-1-producing *K. pneumoniae* significantly decreased ( $P < 0.01$ ), from 7.54% in 2003 to 2.72% in 2004. The results of random amplified polymorphic DNA analysis indicate that the prevalences of DHA-1-producing *K. pneumoniae* and CMY-2-producing *E. coli* strains were not due to epidemic strains. In conclusion, DHA-1 was the most prevalent acquired AmpC beta-lactamase in this collection of isolates from a medical center in China, and DHA-1-producing *K. pneumoniae* was the most prevalent bacterium harboring a PABL. To the best of our knowledge, this is the first report of CMY-2-type AmpC  $\beta$ -lactamases in the Chinese mainland.

Plasmid-mediated AmpC  $\beta$ -lactamases (PABLs) are derived from chromosomal *ampC* genes of the family *Enterobacteriaceae*, such as those of *Citrobacter freundii*, *Enterobacter cloacae*, and *Aeromonas* species.

PABLs have been reported to occur in the United States, Korea, Japan, etc. (4, 14, 24), but there are seldom data indicating PABLs in the Chinese mainland (28, 29). The aims of this study were to establish the prevalence rate of this resistance mechanism in isolates of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Escherichia coli* from a university hospital in Shanghai, China. The first identification of the CMY-2 AmpC  $\beta$ -lactamase in the Chinese mainland is also described.

## MATERIALS AND METHODS

**Bacterial isolates.** A total of 1,935 nonduplicate clinical isolates of *Escherichia coli* ( $n = 1,203$ ), *Klebsiella pneumoniae* ( $n = 699$ ), and *Klebsiella oxytoca* ( $n = 33$ ) were consecutively selected from the Sixth Affiliate Hospital of Shanghai Jiao Tong University between January 2003 and July 2005. Positive controls were used, including *Klebsiella pneumoniae* (strain MISC 304, containing MIR-1) and clinical isolates of *C. freundii*, *E. cloacae*, *Hafnia alvei*, and *Morganella morganii*. Identification was performed by the GNI card of the Vitek system (BioMérieux, MO) or Walk Away 40 (Dade-Bering, Sacramento, CA).

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**Antimicrobial susceptibility testing and screening of  $\beta$ -lactamases.** Isolates were tested for susceptibility by the standard disk diffusion method, and the results were interpreted according to the guidelines of the CLSI (formerly NCCLS). Isolates with cefoxitin zone diameters less than 18 mm (4) were considered positive for the AmpC  $\beta$ -lactamase screening test and were selected for MIC, isoelectric focusing (IEF), PCR, and sequencing analyses. The MICs and the presence of extended-spectrum  $\beta$ -lactamases (ESBLs) were determined by using the ESBL confirmation panel (Dade-Behring, Sacramento, CA). The presence of ESBLs was also investigated by the CLSI-recommended disk screening and confirmatory tests.

**PCR and sequencing.** Multiplex PCR was performed to amplify six PABL group genes (16, 17). A series of specific primers were used for the detection of DHA-1, CMY-2, MOX, ACC, EBC, FOX, TEM, SHV, and CTX-M (7, 16, 23, 27) (Table 1). The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and cloned into DH5 $\alpha$ . Plasmid DNA was prepared by using Qiagen columns and was sequenced on an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA).

**IEF.** IEF was performed as described previously (9). Crude beta-lactamase extracts were subjected to analytical IEF on an ampholine polyacrylamide gel (pH 3.5 to 9.5; Pharmacia, Uppsala, Sweden). Preparations from standard strains known to harbor TEM-12 (pI 5.25), TEM-10 (pI 5.6), TEM-29 (pI 6.1), SHV-1 (pI 7.6), and ACT-1 (pI 9) plasmid-mediated  $\beta$ -lactamases were used as controls. Beta-lactamases were visualized with a 0.2-mg/ml nitrocefin solution (Oxoid Ltd., Basingstoke, England). A 1 mM solution of potassium clavulanate and a 0.3 mM solution of cloxacillin were used to visualize general inhibitor characteristics.

**DNA fingerprinting.** Random amplified polymorphic DNA (RAPD) analysis was applied to type the PABL-producing isolates with the primer ERIC2. Amplified PCR products were separated using 1.5% agarose gels and visualized by UV transillumination. DNA fingerprints were compared by visual inspection. RAPD patterns were regarded as different if there were different bands on visual inspection (10, 12, 21).

**Conjugation.** *E. coli* C600, with rifamycin resistance (Rif<sup>r</sup>), was used as the recipient strain. Cultures of donor and recipient cells were grown to saturation, and 0.1 ml of each was added to the same medium (5 ml) and allowed to stand

TABLE 1. Primers used for PCR of *bla* genes

Target	Primer	Sequence <sup>a</sup> (5' to 3' as synthesized)	Product size (bp)	Annealing temp (°C)	Reference
CIT	CIT-MF	TGG CCA GAA CTG ACA GGC AAA	462	64	17
	CIT-MR	TTT CTC CTG AAC GTG GCT GGC			
DHA	CMY25F1	CAA TGT GTG AGA AGC AGT C	1,432	50	7
	CMY2DR1	CGC ATG GGA TTT TCC TTG CTG			
DHA	DHA-MF	AAC TTT CAC AGG TGT GCT GGG T	405	64	17
	DHA-MR	CCG TAC GCA TAC TGG CTT TGC			
DHA	DHA-1A	TGA TGG CAC AGC AGG ATA	998	52	27
	DHA-1B	GGC TTT GAC TCT TTC GGT A			
MOX	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520	64	17
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C			
ACC	ACCMF	AAC AGC CTC AGC AGC CGG TTA	346	64	17
	ACCMR	TTC GCC GCA ATC ATC CCT AGC			
EBC	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302	64	17
	EBCMR	CTT CCA CTG CGG CTG CCA GTT			
FOX	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190	64	17
	FOXMR	CAA AGC GCG TAA CCG GAT TGG			
TEM	TEM-F	TCA ACA TTT CCG TGT CG	860	42	13
	TEM-R	CTG ACA GTT ACC AAT GCT TA			
SHV	SHV-F	ATG CGT TAT ATT CGC CTG TG	780	47	13
	SHV-R	AGA TAA ATC ACC ACA ATG CGC			
CTX	CTXMU1	ATG TGC AGY ACC AGT AAR GT	593	50	16
	CTXMU2	TGG GTR AAR TAR GTS ACC AGA			

<sup>a</sup> R, A or G; M, A or C; Y, C or T; K, G or T; S, G or C; I, inosine.

at 37°C for 2 h. The mixture was then incubated with shaking for 3 h, and 0.1-ml samples were streaked onto MacConkey agar containing 100 mg/liter rifampin and 10 mg/liter cefotaxime.

**Southern hybridization.** Southern blot hybridizations were performed by standard methods (22) with a *bla*<sub>CMY-2</sub>-specific and *bla*<sub>DHA-1</sub>-specific digoxigenin (DIG)-labeled probe. Briefly, purified plasmid DNA was transferred onto a positively charged nylon membrane (Millipore) by capillary action. DIG-labeled *bla*<sub>CMY-2</sub>-specific and *bla*<sub>DHA-1</sub>-specific detection probes were generated according to the directions of the manufacturer (Roche Diagnostics). After prehybridization, hybridization of the membrane with the denatured, DIG-labeled probe (25 ng/ml of hybridization solution) was done overnight at 65°C. The hybridized probes were immunodetected with anti-DIG-alkaline phosphatase.

**Statistical analysis.** All statistical tests were two-tailed  $\chi^2$  tests, and the results were considered statistically significant at a *P* value of <0.05. The data were stored and analyzed by using SPSS (version 12.0).

## RESULTS

Three hundred twenty-seven (16.9%) of the 1,935 clinical isolates yielded cefoxitin zone diameters less than 18 mm (screen positive), 54 of which were demonstrated to harbor PABLs by multiplex PCR; 41 isolates carried *bla* genes of the DHA group, and 13 isolates carried genes belonging to the CIT group.

Two types of PABLs were detected by PCR with specific primers and confirmed as DHA-1 (41 isolates) and CMY-2 (13 isolates) by sequencing analysis. Each isolate produced one to four  $\beta$ -lactamases, with pI values of 5.4, 7.6, 8.2, 7.8, and 9.0. The pI 5.4  $\beta$ -lactamase corresponds to the TEM-like enzyme

(inhibited by clavulanate), and the pI 7.6 and 8.2  $\beta$ -lactamases correspond to SHV-like and CTX-like enzymes (inhibited by clavulanate), respectively. The  $\beta$ -lactamases with pI values of 7.8 and 9.0 were inhibited by cloxacilin but not by clavulanate and were consistent with DHA-1 and CMY-2 enzymes, respectively.

Based on species, PABLs were detected in 30 (4.29%) of 699 *K. pneumoniae*, 23 (1.91%) of 1,203 *E. coli*, and 1 (3.03%) of 33 *K. oxytoca* isolates. The genotypes of PCR amplification showed that DHA-1 and CMY-2 were harbored by 29 and 1 of 30 PABL-producing *K. pneumoniae* isolates and 11 and 12 of the 23 *E. coli* isolates, respectively. The prevalence rate of DHA-1-producing *K. pneumoniae* was 7.54% in 2003, and this proportion significantly decreased, to 2.72%, in 2004 (*P* < 0.01) but was not significant between 2003 and 2005 (2.96%) (Table 2). ERIC2 PCR was performed to investigate whether the prevalence of DHA-1-producing *K. pneumoniae* was due to nosocomial outbreaks of infections caused by epidemic strains. The 15 isolates of DHA-1-producing *K. pneumoniae* from 2003 had seven different patterns (Fig. 1). Two of the DHA-1-producing *K. pneumoniae* isolates from 2004 displayed the same RAPD pattern. Only 5 of 13 DHA-1-producing *E. coli* strains showed one pattern (data not shown). Two CMY-2-producing *E. coli* isolates from 2003 and six from 2005 indicated very similar RAPD patterns, while four isolates from 2004 presented different types.

Twenty-seven DHA-1-producing isolates and 10 CMY-2-

TABLE 2. Prevalence of plasmid-mediated AmpC-producing clinical isolates in a Chinese university hospital from 2003 to 2005

Organism and yr	No. of isolates				
	Total	Nonsusceptible to cefoxitin	Multiplex PCR positive	Producing indicated AmpC $\beta$ -lactamase	
				DHA-1	CMY-2
<i>E. coli</i>					
2003	400	75	7	5	2
2004	513	88	7	3	4
2005	290	79	9	3	6
Total	1,203	242	23	11	12
<i>K. pneumoniae</i>					
2003	199	30	15	15	0
2004	331	31	10	9	1
2005	169	23	5	5	0
Total	699	84	30	29	1
<i>K. oxytoca</i>					
2003	16	1	1	1	0
2004	11	0	0	0	0
2005	6	0	0	0	0

producing isolates were positive for ESBL production according to phenotypic ESBL confirmatory tests (Table 3). Based on species, 78.3% (18/23) of *E. coli* strains and 60% (18/30) of *K. pneumoniae* strains produced PABL and ESBL simultaneously. In order to determine the ESBL gene relatively exactly, we analyzed the beta-lactamases of these isolates by PCR experiments with a series of primers specific for *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub>. All 54 PABL-producing isolates harbored *bla*<sub>TEM</sub>-like genes. *bla*<sub>SHV</sub>-like and *bla*<sub>CTX-M</sub>-like genes were found in 37 and 29 of the 54 isolates, respectively. Thirty-seven of 41 DHA-1-producing isolates harbored *bla*<sub>SHV</sub>-like genes, and 20 of these 37 isolates harbored *bla*<sub>CTX-M</sub>-like genes simultaneously. Nine of 13 CMY-2-producing isolates harbored *bla*<sub>CTX-M</sub>-like genes. Four of 13 CMY-2-producing isolates transferred *bla*<sub>CMY-2</sub> to transconjugants, 3 had *bla*<sub>CMY-2</sub> alone, and 1 had *bla*<sub>CMY-2</sub> and *bla*<sub>CTX-M</sub>. The Southern blot

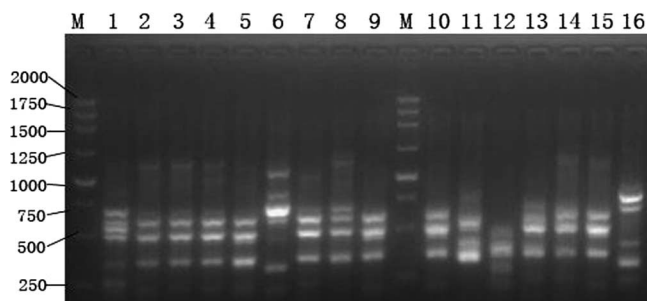


FIG. 1. RAPD fingerprinting of isolates investigated in this work, carried out with primer ERIC2. Lanes 1 to 15, the 15 isolates of DHA-1-producing *K. pneumoniae* from 2003; lane 16, ATCC 25922; M, 250-bp ladder.

TABLE 3. Prevalence of AmpC and ESBL genes and correlation with phenotypes from ESBL tests

Species and $\beta$ -lactamase	No. of isolates <sup>a</sup>					
	Harboring genes in indicated group				Positive for ESBL production	Cef <sup>r</sup>
	AmpC	TEM	CTX-M	SHV		
<i>E. coli</i>						
DHA-1	11	11	5	8	8	8
CMY-2	12	12	9	0	10	6
<i>K. pneumoniae</i>						
DHA-1	29	29	15	28	18	11
CMY-2	1	1	0	0	0	0
<i>K. oxytoca</i>						
DHA-1	1	1	0	1	1	0
Total	54	54	29	37	37	25

<sup>a</sup> Cef<sup>r</sup> represents resistance to cefepime.

analysis further revealed that *bla*<sub>DHA-1</sub> (data not shown) and *bla*<sub>CMY-2</sub> were located on plasmids (Fig. 2).

The susceptibilities of the 54 isolates were as follows: piperacillin, 0%; cefazolin, 0%; cefotaxime, 13%; ceftazidime, 25.9%; cefepime, 53.7%; imipenem, 98%; and meropenem, 98%. The MICs and pI values of the isolates harboring CMY-2-type PABLs are given in Table 4.

## DISCUSSION

Plasmid-mediated class C  $\beta$ -lactamases have been discovered most frequently in naturally AmpC-negative species, such as *K. pneumoniae*, *K. oxytoca*, *Salmonella* spp., and *Proteus mirabilis*, and also in *E. coli*, which normally weakly expresses the chromosomal AmpC enzyme by gene duplication or mutation in the *ampC* promoter or attenuator, with consequent enhanced gene expression. Since CMY-1 was first reported (isolated from South Korea in 1989), over 40 types of PABLs have been reported worldwide. The prevalence of PABLs among clinical isolates differs depending on the countries and institutions. In China, though PABL (ACT-1 type) was first reported in 2002 (3), the true rate of occurrence of PABLs remains unknown. In this study, the prevalence rate (2.79%) of PABLs in *Escherichia coli* and *Klebsiella* spp. is similar to that in Korea (3.1%) but higher than that in the United States (1.2%) (4, 25). The inducible PABL DHA-1 is the most prevalent PABL in China. DHA-1 is also the most prevalent PABL in other areas of Asia (25, 30).

Based on species, 1.9% of *E. coli* and 4.3% of *K. pneumoniae* isolates harbored PABLs in this study. In Korea, the occurrence rates for these species are 1.5% and 5.4% (or 2.9%), respectively (24, 25). But in the United States, they decreased to 1.6% (4) and 3.3% (13), respectively. The prevalence rate of DHA-1-producing *K. pneumoniae* significantly decreased, from 7.54% to 2.72%, between 2003 and 2004 in this study. In contrast, it significantly increased in Korea, from 0.6% in 2002 to 2.8% in 2003 and to 4.3% in 2004 (24). The results of RAPD analysis indicate that the prevalences of DHA-1-producing *K. pneumoniae* (Fig. 1) and CMY-2-producing *E. coli* strains were

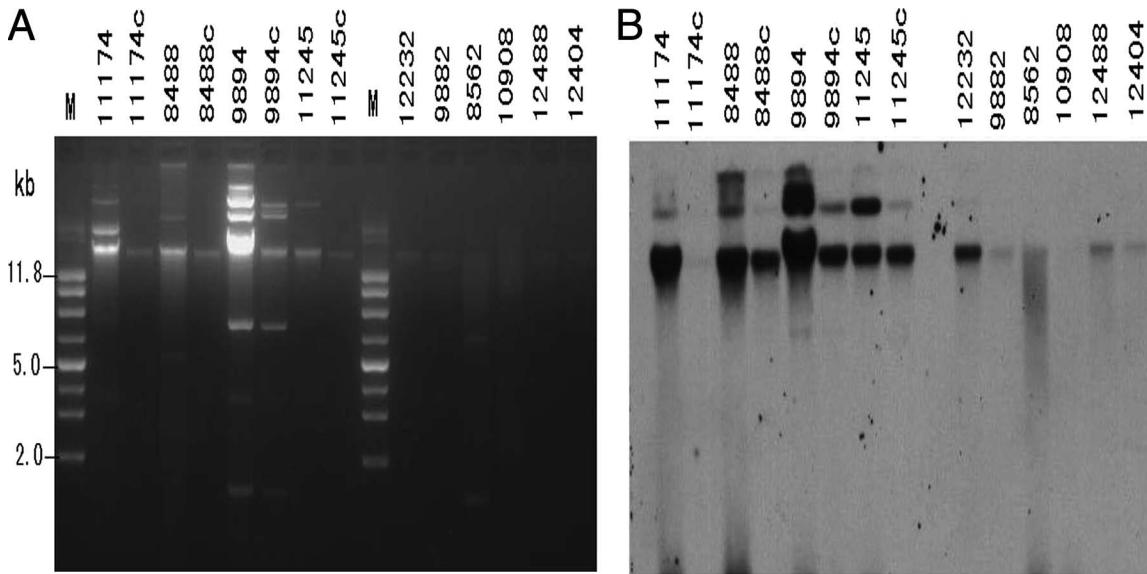


FIG. 2. Plasmid profiles and Southern blotting. (A) plasmid DNA prepared from CMY-2-producing isolates and their transconjugants. Lane M, molecular size marker. (B) Southern blotting of the plasmid DNAs in panel A with the *bla*<sub>CMY-2</sub> probe. The two photographs were aligned so that the size marker can apply to both.

due to a combination of clonal spread and horizontal transmission of plasmids (Fig. 3).

Because PABLs provided a broader spectrum of resistance than ESBLs and were not inhibited by commercially available inhibitors, the antimicrobial agents that could be used are limited. Previous studies suggested that cefepime might be effective for the treatment of infections caused by an AmpC-producing organism. However, in this study, only 53.7% (29 of 54) PABL producers were susceptible to cefepime. Song et al. reported that the association of PABLs with ESBLs may cause failure of treatment, and there is also a report indicating the high inoculum effect of cefepime in PABL-producing isolates (11, 20, 26). Thirty-seven (68.5%) of the 54 PABL-producing isolates also produced an ESBL, which was confirmed by CLSI-recommended confirmatory tests. By the PCR method, 47 (87.0%) of the 54 isolates were detected to harbor *bla*<sub>CTX</sub>-

like or *bla*<sub>SHV</sub>-like genes. Based on species, 93.3% (28/30) of *K. pneumoniae* and 78.3% (18/23) of *E. coli* isolates harbored beta-lactamase genes of AmpC and *bla*<sub>CTX</sub>-like or *bla*<sub>SHV</sub>-like genes simultaneously. This rate of *K. pneumoniae* is far higher than that in Korea (8.7%) but is lower than that in Japan (100%) (14, 24). In this study, one isolate (no. 12404), which harbored *bla*<sub>CMY-2</sub>, *bla*<sub>TEM</sub>-like, and *bla*<sub>CTX</sub>-like genes, was simultaneously resistant to imipenem and meropenem. Poirel et al. reported that one *E. coli* strain with CMY-2 and without outer membrane proteins *OmpF* and *OmpC* was resistant to carbapenem and other broad-spectrum cephalosporins (19). The conjugation test of this isolate was not successful, and further research is in progress.

Southern blot hybridization revealed that the CMY-2 gene is located on plasmid. The different bands that hybridized to the CMY-2 probe, though the size of each band differed in each

TABLE 4. Results for IEF and susceptibility testing of clinical isolates carrying the CMY-2-type *bla*<sub>AmpC</sub> gene

Strain	pI values	Organism <sup>b</sup>	MIC (μg/ml) <sup>a</sup> for:										
			IMP	CTN	MER	CPE	CAZ	CFT	CFX	PI	AZT	CPD	CAX
8488	5.4, 8.2, 9.0	ECO	≤0.5	32	≤0.5	>32	32	>128	>32	>64	64	>64	>64
8562	5.4, 9.0	ECO	≤0.5	32	≤0.5	2	32	8	>32	>64	16	>64	16
9882	5.4, 9.0	KPN	≤0.5	32	≤0.5	<1	16	4	>32	>64	16	>64	16
9894	5.4, 7.6, 9.0	ECO	≤0.5	>32	≤0.5	>32	>128	>128	>32	>64	>64	>64	>64
10908	5.4, 9.0	ECO	≤0.5	4	≤0.5	<1	8	4	32	>64	4	>64	4
11174	5.4, 8.2, 9.0	ECO	≤0.5	>32	≤0.5	>32	64	>128	>32	>64	32	>64	>64
11245	5.4, 8.2, 9.0	ECO	≤0.5	32	≤0.5	>32	128	>128	>32	>64	64	>64	>64
12232	5.4, 8.2, 9.0	ECO	≤0.5	32	≤0.5	>32	128	>128	>32	>64	>64	>64	>64
12404	5.4, 8.2, 9.0	ECO	16	>32	16	>32	>128	>128	>32	>64	>64	>64	>64
12488	5.4, 8.2, 9.0	ECO	≤0.5	32	≤0.5	>32	128	>128	>32	>64	64	>64	>64
12535	5.4, 9.0	ECO	≤0.5	32	≤0.5	>32	128	>128	>32	>64	64	>64	>64
12575	5.4, 8.2, 9.0	ECO	≤0.5	>32	≤0.5	>32	64	>128	>32	>64	>64	>64	>64
13317	5.4, 8.2, 9.0	ECO	≤0.5	32	≤0.5	<1	32	8	>32	>64	8	>64	16

<sup>a</sup> IMP, imipenem; CTN, cefotetan; MER, meropenem; CPE, cefepime; CAZ, ceftazidime; CFT, cefotaxime; CFX, ceftaxitin; PI, piperacillin; AZT, aztreonam; CPD, cefpodoxime; CAX, ceftriaxone.

<sup>b</sup> ECO, *E. coli*; KPN, *K. pneumoniae*.



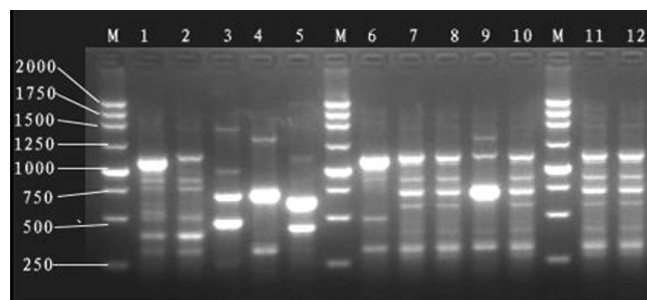


FIG. 3. RAPD fingerprinting of isolates investigated in this work, carried out with primer ERIC2. Lanes 1 to 12, the 12 isolates of CMY-2-producing *E. coli* from 2003 to 2005. M, 250-bp ladder.

isolate, could presumably represent another gene that has homology to *bla*<sub>CMY-2</sub> or partial or complete duplications of CMY-2.

The CMY-2  $\beta$ -lactamase was first described in 1990 (2). It is the ancestor of the other *C. freundii ampC* alleles that have been found on plasmids since then (1). CMY-2 was detected not only in *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis* but also in *Salmonella* and *Shigella* (5, 8, 15), the last two having caused an outbreak. CMY-2 is the most prevalent and geographically the most widely distributed PABL (18). In China, the most prevalent type of PABL is DHA-1. The CMY-2  $\beta$ -lactamase has not been previously reported to occur in China. In 2004, Guan et al. (6) reported a new CMY-type cephalosporinase which is 99% identical to the deduced amino acid sequences of CMY-2 and CMY-7. To the best of our knowledge, this is the first report of plasmid-mediated CMY-2-type AmpC  $\beta$ -lactamases in mainland China.

In conclusion, the prevalence rate of PABLs is 2.79% in *Escherichia coli* and *Klebsiella* spp. in this collection of isolates from a medical center in China. DHA-1 is the predominant PABL, and DHA-1-producing *K. pneumoniae* is the most prevalent bacterial species harboring PABLs. The results of this study reinforce the need for increasing concern for therapy for clinical infections caused by isolates that coproduce PABL and ESBL. This study also represents the first time that CMY-2 has been described to occur in China.

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