Development of a Multiplex PCR and SHV Melting-Curve Mutation Detection System for Detection of Some SHV and CTX-M β -Lactamases of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* in Taiwan

Ju-Hsin Chia,¹ Chishih Chu,³ Lin-Hui Su,¹ Cheng-Hsun Chiu,²
An-Jing Kuo,¹ Chien-Feng Sun,¹ and Tsu-Lan Wu^{1*}

Department of Clinical Pathology, Chang Gung Memorial Hospital,¹ and Department of Pediatrics, Chang Gung Children's Hospital,² Taoyuan, Taiwan, and Department of Applied Microbiology, National Chiayi University, Chiayi, Taiwan³

Received 14 October 2004/Returned for modification 9 December 2004/Accepted 13 June 2005

Infection by extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* has been increasing in Taiwan. Accurate identification of the ESBL genes is necessary for surveillance and for epidemiological studies of the mode of transmission in the hospital setting. We describe herein the development of a novel system, which consists of a multiplex PCR to identify *bla*_{SHV}, *bla*_{CTX-M-3}-like, and *bla*_{CTX-M-14}-like genes and a modified SHV melting-curve mutation detection method to rapidly distinguish six prevalent *bla*_{SHV} genes (*bla*_{SHV-1}, *bla*_{SHV-2}, *bla*_{SHV-2a}, *bla*_{SHV-5}, *bla*_{SHV-11}, and *bla*_{SHV-12}) in Taiwan. Sixty-five clinical isolates, which had been characterized by nucleotide sequencing of the *bla*_{SHV} and *bla*_{CTX-M} genes, were identified by the system. The system was then used to genotype the ESBLs from 199 clinical isolates, including 40 *Enterobacter cloacae*, 68 *Escherichia coli*, and 91 *Klebsiella pneumoniae*, collected between August 2002 and March 2003. SHV-12 (80 isolates) was the most prevalent type of ESBL identified, followed in order of frequency by CTX-M-3 (65 isolates) and CTX-M-14 (36 isolates). Seventeen (9%) of the 199 clinical isolates harbored both SHV- and CTX-M-type ESBLs. In contrast to *Enterobacter cloacae*, the majority of which produced SHV-type ESBLs, *E. coli* and *K. pneumoniae* were more likely to possess CTX-M-type ESBLs. Three rare CTX-M types were identified through sequencing of the *bla*_{CTX-M-3}-like (CTX-M-15) and *bla*_{CTX-M-14}-like (CTX-M-9 and CTX-M-13) genes. The system appears to provide an efficient differentiation of ESBLs among *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae* in Taiwan. Moreover, the design of the system can be easily adapted for similar purposes in areas where different ESBLs are prevalent.

Infection by extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* has become a serious problem in Taiwan. According to previous reports from various local hospitals (7, 10, 15, 25, 27, 28, 30, 32), ESBLs were produced by 8 to 30% and 1.6 to 6.7% of clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli*, respectively. Some ESBL phenotypes, including SHV-2, SHV-2a, SHV-5, SHV-12, CTX-M-3, and CTX-M-14, have been found to be prevalent across Taiwan (15, 25, 27, 28, 30, 32). In Chang Gung Memorial Hospital, a university-affiliated, 4,000-bed hospital located in northern Taiwan, we had found that the emergence and dissemination of ESBL-producing *Enterobacteriaceae* in pediatric intensive care units is due to clonal dissemination of a few epidemic strains as well as horizontal transmission of resistance gene-carrying plasmids among bacterial organisms (27). Our previous study indicated that SHV-12 and CTX-M-3 are the most common ESBLs produced by clinical isolates in Chang Gung Memorial Hospital. However, other ESBLs, such as SHV-2, SHV-2a, SHV-5, and CTX-M-14, are sporadically reported (27).

Rapid detection and identification of ESBLs appear essen-

tial in studying the epidemiology of antimicrobial resistant bacteria. A number of molecular biological methods, such as PCR-restriction fragment length polymorphism (PCR-RFLP) analysis (1, 5, 14), oligotyping (18), PCR single-strand conformational polymorphism (5, 16), and ligase chain reaction (12, 22), have been developed for the detection of SHV and TEM derivatives. Randegger and Hächler reported a rapid and sensitive method, termed SHV melting-curve mutation detection (MCMD), to detect the mutations in three codons at positions 179, 238, and 240 of the *bla*_{SHV} gene in a single reaction (23). Nucleotide sequencing remains the standard method for the determination of specific β -lactamase genes in respective bacterial isolates. However, different ESBLs may be copresented in one organism (6, 8, 27, 28, 31). Multiple PCR-sequencing processes are required in such a setting, and the whole procedure appears time-consuming and nonefficient.

In this study, we described the development of a novel system, which consists of a multiplex PCR to identify *bla*_{SHV}, *bla*_{CTX-M-3}-like and *bla*_{CTX-M-14}-like genes and a modified SHV MCMD method to rapidly distinguish six prevalent *bla*_{SHV} genes (*bla*_{SHV-1}, *bla*_{SHV-2}, *bla*_{SHV-2a}, *bla*_{SHV-5}, *bla*_{SHV-11}, and *bla*_{SHV-12}) in Taiwan. By using this system, clinical isolates of ESBL-producing members of the family *Enterobacteriaceae*, including *Enterobacter cloacae*, *Escherichia coli*, and *Klebsiella pneumoniae*, were also investigated.

* Corresponding author. Mailing address: Department of Clinical Pathology, Chang Gung Memorial Hospital, 5 Fu-Hsin St., Kweishan 333, Taoyuan, Taiwan. Phone: 886 3 3281200, ext. 2736. Fax: 886 3 3971827. E-mail: wutsulan@adm.cgmh.org.tw.

TABLE 1. Primers and probes used for the multiplex PCR and SHV MCMD system

Category	Test	Target	Name	Sequence (5' to 3')	Expected amplicon size (bp)	Nucleotide positions ^c	GenBank accession no.
Primer	Multiplex PCR	<i>bla</i> _{CTX-M-3} -like ^a	CTX-3F	AATCACTGCGCCAGTTACGCT	479	11 to 32	Y10278
			CTX-3R	GAACGTTTCGTCTCCCAGCTGT		489 to 468	
		<i>bla</i> _{CTX-M-14} -like ^b	CTX-14F	TACCGCAGATAATACGCAGGTG	355	162 to 183	AF252622
			CTX-14R	CAGCGTAGGTTCACTGCGATCC		516 to 495	
	SHV MCMD	<i>bla</i> _{SHV}	SHV-F	AACGGAAGTGAATGAGGCGCT	141	486 to 506	AF124984
			SHV-R	TCCACCATCCACTGCAGCAGCT		626 to 605	
Probe	SHV MCMD	<i>bla</i> _{SHV}	F1	GCCTTTATCGGCCCTCACTCAA	819	-37 to -16	AF124984
			A2	TCCCGCAGATAAATCACCACAATG		782 to 759	
		Codon 35 ^d	Sensor T	LCRed 705-CTTTCGCTTAGTTTAATTTGC		101 to 76	AF124984
				TCAAG-p			
			Anchor I	TCATGCCTACGCGGCCCGACAGCT-X		127 to 104	
			Sensor A	GACCGGAGCTAGCAAGCG-X ^e		690 to 707	AF462395
		Codons 238/240 ^d	Anchor II	LCRed 640-TGCGCGCGGGATTGTCGCC-p ^e		711 to 729	

^a *bla*_{CTX-M-3}-like represents the genes encoding CTX-M β-lactamases similar to CTX-M-3, including CTX-M-1, -3, -10 to -12, -15, -22, -23, -28, -29, and -30.

^b *bla*_{CTX-M-14}-like represents the genes encoding CTX-M β-lactamases similar to CTX-M-14, including CTX-M-9, -13, -14, -16 to -19, -21, -24, -27, and Toho-2.

^c The numbering for nucleotide positions is based on positions coordinating to the open reading frame.

^d Both of the *bla*_{SHV} genes.

^e The sequences were adapted from reference 23.

MATERIALS AND METHODS

Bacterial strains. To assess the accuracy of the novel system described herein, a total of 65 clinical isolates, including six *Enterobacter cloacae*, 18 *E. coli*, and 41 *K. pneumoniae*, the ESBL genotypes of which had been identified by conventional nucleotide sequencing methods, were used in the preliminary stage. To ascertain that accurate results can be yielded from different ESBL-producing strains, including those produced multiple ESBLs, a half of the 65 isolates were selected from those able to produce SHV- and CTX-M-type β-lactamases simultaneously, while the other half produced only SHV ($n = 23$) or CTX-M ($n = 10$) enzymes (see Table 2).

The applicability of the system was further evaluated by using a collection of 199 clinical isolates, including 40 *Enterobacter cloacae*, 68 *E. coli*, and 91 *K. pneumoniae*. These isolates were collected from patients in various intensive care units of this hospital between August 2002 and March 2003 and had been confirmed to be ESBL-producers by the phenotypic confirmatory test recommended by the National Committee for Clinical Laboratory Standards (now called the Clinical and Laboratory Standards Institute) (20, 21).

DNA preparation. A loopful of bacteria colonies harvested from a blood agar plate was suspended in 0.5 ml of sterile water and heated at 95°C for 10 min. After centrifugation at 5,000 rpm for 5 min at 4°C, the DNA-containing supernatant was used as the source of template for further amplification.

Multiplex PCR and SHV MCMD system. The system consists of a multiplex PCR and a modified SHV MCMD method. The multiplex PCR was developed to identify *bla*_{SHV}, *bla*_{CTX-M-3}-like, and *bla*_{CTX-M-14}-like genes simultaneously. Following the multiplex PCR, the specific SHV and CTX genotypes were further identified, respectively, by a SHV MCMD method described by Randegger and Hächler with some modifications and a conventional nucleotide sequencing method.

Three different DNA targets (141, 479, and 355 base pairs) within the genes of *bla*_{SHV}, *bla*_{CTX-M-3}-like, and *bla*_{CTX-M-14}-like were amplified simultaneously. Their oligonucleotide sequences are listed in Table 1. The specificities of these primer sets have been confirmed by BLAST search in GenBank. The two primer sets, CTX-3F/3R and CTX-14F/14R, were designed to amplify the two groups of CTX-M enzymes similar to CTX-M-3 (CTX-M-1, -3, -10 to -12, -15, -22, -23, -28, -29, and -30) and CTX-M-14 (CTX-M-9, -13, -14, -16 to -19, -21, -24, and -27 and Toho-2), respectively. Isolates with positive PCR results were subjected for further PCR and sequencing analysis to identify the specific CTX-M types using methods described below.

Amplification was performed in a DNA thermal cycler (Perkin-Elmer Biosystems, Foster City, Calif.) in a 50-μl mixture containing 1.5 U *Taq* DNA polymerase (ABgene, Epsom, Surrey, United Kingdom), 200 μM each of the four deoxynucleoside triphosphates (GeneTeks BioScience, Inc., Taipei, Taiwan), and 0.6 μM of each oligonucleotide primer in 1x PCR buffer. Template DNA (2 μl) was added to 48 μl of the master mixture and then overlaid with mineral oil. Amplification profile included an initial denaturation step at 94°C for 2 min and then 35 cycles with denaturation at 94°C for 1 min, primer annealing at 62°C for

1 min, and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was performed and the products were stored at 4°C until used. After gel electrophoresis, the ethidium bromide-stained PCR products were visualized under UV light.

A subsequent SHV MCMD method was used when the presence of *bla*_{SHV} was indicated by the multiplex PCR. The forward primer F1 and the reverse primer A2 were used to amplify an 819-bp PCR product of the *bla*_{SHV} coding region spanning nucleotide position -37 to +782 of the strain with GenBank accession no. AF124984 (Table 1). To specifically identify possible SHV variants, two hybridization probes were used concomitantly in a single reaction tube. The first one was designed by Randegger and Hächler (23), and consisted of two oligonucleotide probes, sensor A and anchor II, which were labeled with LightCycler Red 640 dye (measured at 640 nm in channel F2). Another unique hybridization probe consisted of probes sensor T and anchor I were labeled with LightCycler Red 705 dye (measured at 710 nm in channel F3). The oligonucleotide sequences of these probes are listed in Table 1. The sequences of probes sensor T and anchor I were designed to fit *bla*_{SHV-1} without any mutation at position 35, and thus were used to detect the mutation at codon 35. Another probes (sensor A and anchor II) were used to detect the mutations at codons 238 and 240. The sequences were designed to fit *bla*_{SHV-12} with a double mutation at positions 238 and 240. Both hybridization probes were synthesized by TIB MOL-BIOL (Berlin, Germany).

PCR was performed by rapid cycling in a reaction volume of 10 μl with each amplification primer at a concentration of 0.25 μM and each detection and anchor probe at a concentration of 0.1 μM. LightCycler FastStart DNA Master Hybridization Probe Buffer (Roche Molecular Biochemicals, Mannheim, Germany) was used; 9 μl of the master mixture and 1 μl of a DNA preparation were loaded into glass capillary cuvettes (Roche Molecular Biochemicals, Mannheim, Germany). After a short centrifugation (3,000 × g for 10 s), the sealed capillaries were placed into the LightCycler rotor. After an initial polymerase activation and denaturation step at 95°C for 7 min, the samples run 50 amplification cycles, each comprising denaturation (95°C for 10 s), annealing (60°C for 10 s), and extension (72°C for 38 s) in the LightCycler instrument.

Fluorescence was measured at the end of the annealing period of each cycle to monitor the progress of amplification. After completion, a melting curve was recorded by cooling to 35°C at 20°C/s, holding at 35°C for 15s, and then heating slowly at 0.1°C/s until 85°C. Fluorescence was measured continuously during the slow temperature rise to monitor dissociation of the LightCycler Red 640-labeled detection probe at F2 and the LightCycler Red 705-labeled detection probe at F3. Fluorescence signals were plotted automatically in real time versus temperature (T) to produce melting curves for mutations at position 35 (F3 versus T) and positions 238 and 240 (F2 versus T). Melting curves were then converted into melting peaks by plotting the negative derivative of fluorescence versus T ($-dF2/dT$ versus T and $-dF3/dT$ versus T).

PCR amplification and DNA sequencing. To determine the ESBL types, two previously described primer sets were used to detect *bla*_{SHV} and *bla*_{CTX-M-14}-like

FIG. 1. Electrophoresis of the amplified products of *bla*_{SHV}, *bla*_{CTX-M-3-like}, and *bla*_{CTX-M-14-like} genes by a multiplex PCR in a 2% agarose gel. Lane M, 100-bp DNA ladder; lanes 1 to 5, clinical isolates. The amplified products and their sizes are indicated on the right, and the sizes of the markers in base pairs are shown on the left.

genes in the amplification procedure (17, 24). One novel primer sets, CTX-F (5'-TCCCAGATAAGGAATCCCAT-3') and CTX-R1 (5'-CCCATTCCGTT TCCGCTA-3'), were constructed to detect *bla*_{CTX-M-3-like} genes. Primers CTX-F and CTX-R1 corresponding to nucleotide positions -19 to +2 and +894 to +877, respectively, of the *bla*_{CTX-M-3} gene sequence with GenBank accession no. AF550415. PCR products were then subjected for DNA sequencing as described previously (27).

RESULTS

Assessment of accuracy of the multiplex PCR and SHV MCMD system. Fig. 1 shows the results of the multiplex PCR. Isolates with multiple β -lactamases can be demonstrated clearly by the multiplex PCR as shown in lanes 1, 3, and 5 (Fig. 1). The results of SHV MCMD are demonstrated in Fig. 2. Fluorescence F3 (Fig. 2A) was generated by the probes anchor I and sensor T which targeted codon 35 and thus reveal a higher melting temperature (T_m) for ESBL genes showing no mismatch (*bla*_{SHV-1}, *bla*_{SHV-2}, and *bla*_{SHV-5}) and a lower T_m for those with one mismatch (*bla*_{SHV-2a}, *bla*_{SHV-11}, and *bla*_{SHV-12}) compared with sensor T. Fluorescence F2 (Fig. 2B) was generated by the probes anchor II and sensor A, which targeted codons 238/240. For ESBL genes (*bla*_{SHV-5} and *bla*_{SHV-12}) showing no mismatch with sensor A, the T_m was higher at 64°C, while those with one mismatch (*bla*_{SHV-2} and *bla*_{SHV-2a}) and two mismatches (*bla*_{SHV-1} and *bla*_{SHV-11}) were detected at lower T_m s, 58°C and below 50°C, respectively.

From melting-curve analysis obtained with the LightCycler Red 705-labeled probes at F3 (Fig. 2A), bacterial strains carrying *bla*_{SHV} genes can be separated into two groups. For each group different *bla*_{SHV} genes can be further differentiated into three groups by their T_m s at F2 (Fig. 2B). For example, SHV-5 had a higher T_m at F3 and so can be differentiated from other ESBL genes with one mismatch. It can be further separated from SHV-1 and SHV-2 at F2 by different T_m s at 64°C, below 50°C, and 58°C, respectively. Comparison of the results between the multiplex PCR and SHV MCMD system and the conventional sequencing method in the detection of various ESBLs is shown in Table 2. All 65 clinical isolates which were characterized by nucleotide sequencing of the *bla*_{SHV} and *bla*_{CTX-M} genes were identified accurately by the multiplex PCR and the SHV MCMD system.

FIG. 2. Melting peaks of *bla*_{SHV} genes in clinical isolates. Panel A, fluorescence detected in F3 was generated by the LightCycler Red 705-labeled hybridization probe, which was designed to detect the mutation at codon 35. Melting peaks at higher T_m s (T_m of 66°C) indicated strains harboring *bla*_{SHV} genes without mismatch with the sensor T, including *bla*_{SHV-1}, *bla*_{SHV-2}, and *bla*_{SHV-5}. Lower T_m s indicated those harboring *bla*_{SHV-2a}, *bla*_{SHV-11}, and *bla*_{SHV-12}, the representatives with a mutation at codon 35 (T_m of 62°C). Panel B, fluorescence detected in F2 was generated by the LightCycler Red 640-labeled hybridization probe which was designed to detect mutations at codons 238 and 240. Three melting peaks were demonstrated by the three different T_m s: below 50°C for strains harboring *bla*_{SHV-1} and *bla*_{SHV-11} (two mismatches), 58°C for strains harboring *bla*_{SHV-2}, and *bla*_{SHV-2a} (one mismatch), and 64°C for the strains harboring *bla*_{SHV-5} and *bla*_{SHV-12} (no mismatch).

Investigation of clinical isolates by the multiplex PCR and SHV MCMD system. Specification of SHV- and CTX-M-types among the 199 ESBL-producing isolates is summarized in Table 3. By the multiplex PCR, 142 (72%) were positive for *bla*_{SHV} genes. After SHV MCMD analysis, 108 (76%) of the 142 isolates were shown to harbor *bla*_{SHV-2} ($n = 3$), *bla*_{SHV-2a} ($n = 3$), *bla*_{SHV-5} ($n = 22$), and *bla*_{SHV-12} ($n = 80$) genes. Other β -lactamase genes, such as *bla*_{SHV-1} ($n = 9$) and *bla*_{SHV-11} ($n = 25$), were found in the remaining 34 isolates (24%); their ESBL phenotypes were due to the production of CTX-M-type β -lactamase as identified by the multiplex PCR and the subsequent sequencing analysis. A total of 57 isolates were demonstrated by the multiplex PCR to harbor only *bla*_{CTX-M} but not *bla*_{SHV}. By further sequencing analysis, their genotypes were confirmed to consist of CTX-M-3 (28, 49%), CTX-M-14 (23, 40%), CTX-M-9 (4, 7%), and CTX-M-13 (2, 4%).

TABLE 2. Comparison of multiplex PCR and SHV melting-curve mutation detection system with conventional nucleotide sequencing method in the detection of various ESBLs

ESBL type ^a (no.)	No. of isolates carrying the indicated ESBL characterized by:								
	Multiplex PCR			SHV melting-curve mutation detection					
	SHV	CTX-M-3-like	CTX-M-14-like	SHV-1	SHV-11	SHV-2	SHV-2a	SHV-5	SHV-12
SHV-2a (5)	5	0	0	0	0	0	5	0	0
SHV-5 (3)	3	0	0	0	0	0	0	3	0
SHV-12 (15)	15	0	0	0	0	0	0	0	15
CTX-M-3 (7)	0	7	0			ND ^b			
CTX-M-14 (3)	0	0	3			ND			
SHV-1/CTX-M-3 (5)	5	5	0	5	0	0	0	0	0
SHV-1/CTX-M-14 (1)	1	0	1	1	0	0	0	0	0
SHV-2/CTX-M-3 (3)	3	3	0	0	0	3	0	0	0
SHV-2a/CTX-M-3 (2)	2	2	0	0	0	0	2	0	0
SHV-5/CTX-M-3 (2)	2	2	0	0	0	0	0	2	0
SHV-11/CTX-M-3 (8)	8	8	0	0	8	0	0	0	0
SHV-11/CTX-M-14 (1)	1	0	1	0	1	0	0	0	0
SHV-12/CTX-M-3 (9)	9	9	0	0	0	0	0	0	9
SHV-12/CTX-M-14 (1)	1	0	1	0	0	0	0	0	1

^a The ESBL types were determined by the conventional nucleotide sequencing method.^b ND, not done.

SHV-12 (80 isolates) was the most prevalent type of ESBL identified, followed in order of frequency by CTX-M-3 (65 isolates) and CTX-M-14 (36 isolates). Seventeen (9%) of the 199 clinical isolates, including three *Enterobacter cloacae*, five *E. coli*, and nine *K. pneumoniae*, harbored both SHV- and CTX-M-type ESBLs. In contrast to *Enterobacter cloacae*, the majority of which produced SHV-type ESBLs, *E. coli* and *K. pneumoniae* were more likely to possess CTX-M-type ESBLs ($P < 0.000005$). Three rare CTX-M types which were not present in the previous period during assessment were identified through sequencing of the *bla*_{CTX-M-3-like} (CTX-M-15) and *bla*_{CTX-M-14-like} (CTX-M-9 and CTX-M-13) genes, respectively.

DISCUSSION

Correct identification of the genes involved in ESBL-mediated resistance is necessary for surveillance and epidemiological studies of their transmission in hospitals. In the early studies of ESBLs, isoelectric focusing analysis was usually used to characterize β -lactamases. However, many β -lactamases possess identical isoelectric points and the determination of isoelectric points was equivocal and could be technically demanding. Specific identification of ESBLs by isoelectric focusing analysis may not be efficient.

Recently, a number of molecular biological methods have been proposed for the identification of TEM and SHV derivatives (4, 9). These methods, such as oligotyping (18, 26), PCR-single-strand conformational polymorphism (5, 16),

PCR-restriction fragment length polymorphism (1, 5, 14), and ligase chain reaction (12, 22), usually detect only one type of ESBL each time. In areas such as our institution, which has seen a rapid increase in various ESBL types in the past few years, these methods appear cumbersome if various ESBLs are to be identified.

In contrast, our design reported here provides the first detection system for the specific and simultaneous identification of SHV and CTX-M type ESBLs by using a combination of a multiplex PCR plus a real-time PCR and melting-curve analysis. Moreover, the multiplex PCR and SHV MCMD system offers many advantages. First, the multiplex PCR can be used to screen a large number of clinical isolates producing SHV β -lactamases and a majority of CTX-M-type ESBLs. Second, two primer sets specific to *bla*_{CTX-M-3-like} and *bla*_{CTX-M-14-like} genes allow differentiation of CTX-M-3-like and CTX-M-14-like ESBLs, without sequencing. Finally, SHV MCMD using a LightCycler instrument, without postamplification procedures such as restriction enzyme digestion or gel electrophoresis, provides the advantage of acquiring the results within 1 h. Therefore, we believe that the multiplex PCR and SHV MCMD system may become an important tool for the detection and distinction of SHV and CTX-M β -lactamases.

Since the present study is the first attempt to develop such a novel system to replace the conventional sequencing method, the initial multiplex PCR designed for the simultaneous detection of different types of ESBLs was focused only on the detection of SHV- and CTX-M-type ESBLs, which were the most

TABLE 3. Number and distribution of various ESBLs in strains of *Enterobacter cloacae*, *E. coli*, and *K. pneumoniae*

Bacteria	Total no. examined	No. of isolates producing ESBL:								
		SHV-2	SHV-2a	SHV-5	SHV-12	CTX-M-3	CTX-M-9	CTX-M-13	CTX-M-14	CTX-M-15
<i>Enterobacter cloacae</i>	40	0	0	0	35	8	0	0	0	0
<i>E. coli</i>	68	0	0	5	15	24	4	2	23	0
<i>K. pneumoniae</i>	91	3	3	17	30	33	0	0	13	1

prevalent ESBLs in Taiwan. However, TEM-mediated ESBL production is a major concern for many geographic regions. Further development of simultaneous detection of TEM-, SHV-, and CTX-M-type ESBLs will definitely be an advance for ESBL-related studies. Furthermore, two groups of CTX-M enzymes, CTX-M-3-like and CTX-M-14-like, were included in the multiplex PCR because they have been predominant in Taiwan (15, 27, 28, 29, 32) as well as many other countries (3). However, it is known that, unlike SHV- or TEM-type ESBLs, CTX-M enzymes are much more complicated and can be subclassified into five groups (3). Further optimization to include the other three groups of CTX-M enzymes deserves further investigation.

There are still possibilities that some minor SHV enzymes may not be correctly identified in the SHV MCMD system, although such situations did not occur among our isolates. Even if it did happen, conclusive results can be obtained by further PCR and sequencing analysis to verify the presence of any specific SHV enzymes.

In the present study, the accuracy of the novel multiplex PCR and SHV MCMD system correlated 100% to the conventional nucleotide sequencing method. Furthermore, to test the applicability of the system, 199 ESBL-producing isolates were also examined. By the multiplex PCR, 142 isolates showed positive for *bla*_{SHV} and their genotypes can be further specified by the SHV MCMD. The remaining 57 isolates were positive for *bla*_{CTX-M} only, and the specific CTX-M genes were also identified by the subsequent sequencing analysis. The absence of SHV genes among these 57 isolates was also verified by examination of their MICs and PCR and the Southern blotting method specific for *bla*_{SHV} (data not shown). The results indicated that the identification of SHV and CTX-M β -lactamases from clinical isolates was feasible.

In Chang Gung Memorial Hospital, a retrospective review of laboratory records revealed a rapid increase in the number of ESBL-producing gram-negative isolates since 2000. The prevalence rates of ESBL producers among *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae* isolates have accounted for 5%, 15%, and 23% of the bacterial population, respectively, in 2001. Moreover, the proportion of ESBL-producing isolates was even higher (15%, 25%, and 37%, respectively) in intensive care units than elsewhere. SHV-12 remained the most prevalent type of ESBL among *Enterobacter cloacae*, which was in agreement with previous findings (13, 27).

For *K. pneumoniae*, previous reports from Taiwan indicated that SHV-5-type ESBLs (SHV-5 and -12) were the most prevalent ESBLs (15, 25, 30). However, we found in the current study that CTX-M-type β -lactamases were the most prevalent ESBLs in this organism. Whether the change was due to the replacement of a specific CTX-M β -lactamases-producing *K. pneumoniae* strain or the spread of *bla*_{CTX-M}-carrying plasmids under the selective pressure of different antimicrobial treatment regimens remains to be studied.

As for *E. coli*, the most common ESBLs were CTX-M-3 and CTX-M-14, a finding similar to previous reports from Taiwan (17, 28, 29).

The prevalence rate of CTX-M-14-producing strains in ESBL-producing *K. pneumoniae* and *E. coli* isolates increased significantly from 2% in 2001 (27) to 23% in the current study ($P < 0.0005$). The relatedness of the increase and the finding

that *bla*_{CTX-M-14} was located on plasmids need further investigation. The remaining sporadic ESBLs were only rarely reported in other countries (2, 6, 11, 19). Due at least in part to the busy international travel between Taiwan and foreign countries, we now see the presence of these rare ESBLs in Taiwan. This also represents a new alert from those ESBL-producing pathogens and requires more intensive monitoring.

In conclusion, we have developed a multiplex PCR for the detection of SHV, CTX-M-3-like, and CTX-M-14-like β -lactamase genes. A further method, the SHV MCMD, was used to discriminate the most prevalent types of SHV ESBLs in Taiwan. The system provides an efficient differentiation of ESBLs in selected species of *Enterobacteriaceae*. Moreover, the design of the system can be easily adapted for similar purposes in areas where different ESBLs are prevalent.

ACKNOWLEDGMENTS

We thank Leung Kei Siu of the National Health Research Institute, Taiwan, for his helpful advice in the revision of the manuscript.

This work was supported in part by grants CMRP798 from the Chang Gung Memorial Hospital and NSC 91-2314-B-182A-081 from the National Science Council, Executive Yuan, Taiwan.

REFERENCES

1. Arlet, G., G. Bami, D. Decre, A. Flippo, O. Gaillot, P. H. Lagrange, and A. Philippon. 1995. Molecular characterisation by PCR-restriction fragment length polymorphism of TEM β -lactamases. *FEMS Microbiol. Lett.* **134**: 203–208.
2. Baraniak, A., J. Fiett, W. Hryniewicz, P. Nordmann, and M. Gniadkowski. 2002. Ceftazidime-hydrolysing CTX-M-15 extended-spectrum β -lactamase (ESBL) in Poland. *J. Antimicrob. Chemother.* **50**:393–396.
3. Bonnet, R. 2004. Growing group of extended-spectrum β -lactamases: the CTX-M enzymes. *Antimicrob. Agents Chemother.* **48**:1–14.
4. Bradford, P. A. 2001. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **14**:933–951.
5. Chanawong, A., F. H. M'Zali, J. Heritage, A. Lulitanond, and P. M. Hawkey. 2000. Characterisation of extended-spectrum β -lactamases of the SHV family using a combination of PCR-single strand conformational polymorphism (PCR-SSCP) and PCR-restriction fragment length polymorphism (PCR-RFLP). *FEMS Microbiol. Lett.* **184**:85–89.
6. Chanawong, A., F. H. M'Zali, J. Heritage, J. H. Xiong, and P. M. Hawkey. 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. *Antimicrob. Agents Chemother.* **46**:630–637.
7. Chang, F. Y., L. K. Siu, C. P. Fung, M. H. Huang, and M. Ho. 2001. Diversity of SHV and TEM β -lactamases in *Klebsiella pneumoniae*: gene evolution in Northern Taiwan and two novel beta-lactamases, SHV-25 and SHV-26. *Antimicrob. Agents Chemother.* **45**:2407–2413.
8. Essack, S. Y., L. M. Hall, D. G. Pillay, M. L. McFadyen, and D. M. Livermore. 2001. Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum β -lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. *Antimicrob. Agents Chemother.* **45**:88–95.
9. Fluit, A. C., M. R. Visser, and F. J. Schmitz. 2001. Molecular detection of antimicrobial resistance. *Clin. Microbiol. Rev.* **14**:836–871.
10. Jan, I. S., P. R. Hsueh, L. J. Teng, S. W. Ho, and K. T. Luh. 1998. Antimicrobial susceptibility testing for *Klebsiella pneumoniae* isolates resistant to extended-spectrum β -lactam antibiotics. *J. Formos. Med. Assoc.* **97**:661–666.
11. Karim, A., L. Poirel, S. Nagarajan, and P. Nordmann. 2001. Plasmid-mediated extended-spectrum β -lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEp1. *FEMS Microbiol. Lett.* **201**:237–241.
12. Kim, J., and H. J. Lee. 2000. Rapid discriminatory detection of genes coding for SHV β -lactamases by ligase chain reaction. *Antimicrob. Agents Chemother.* **44**:1860–1864.
13. Lee, S. H., J. Y. Kim, S. H. Shin, Y. J. An, Y. W. Choi, Y. C. Jung, H. I. Jung, E. S. Sohn, S. H. Jeong, and K. J. Lee. 2003. Dissemination of SHV-12 and characterization of new AmpC-type β -lactamase genes among clinical isolates of *Enterobacter* species in Korea. *J. Clin. Microbiol.* **41**:2477–2482.
14. Lee, S. H., J. Y. Kim, S. H. Shin, S. K. Lee, M. M. Choi, I. Y. Lee, Y. B. Kim, J. Y. Cho, W. Jin, and K. J. Lee. 2001. Restriction fragment length dimorphism-PCR method for the detection of extended-spectrum β -lactamases unrelated to TEM- and SHV-types. *FEMS Microbiol. Lett.* **200**:157–161.
15. Liu, P. Y., J. C. Tung, S. C. Ke, and S. L. Chen. 1998. Molecular epidemi-

- ology of extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* isolates in a district hospital in Taiwan. *J. Clin. Microbiol.* **36**:2759–2762.
16. M'Zali, F. H., J. Heritage, D. M. Gascoyne-Binzi, A. M. Snelling, and P. M. Hawkey. 1998. PCR single strand conformational polymorphism can be used to detect the gene encoding SHV-7 extended-spectrum β -lactamase and to identify different SHV genes within the same strain. *J. Antimicrob. Chemother.* **41**:123–125.
 17. Ma, L., Y. Ishii, F. Y. Chang, K. Yamaguchi, M. Ho, and L. K. Siu. 2002. CTX-M-14, a plasmid-mediated CTX-M type extended-spectrum β -lactamase isolated from *Escherichia coli*. *Antimicrob. Agents Chemother.* **46**:1985–1988.
 18. Mabilat, C., and P. Courvalin. 1990. Development of "oligotyping" for characterization and molecular epidemiology of TEM β -lactamases in members of the family *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* **34**:2210–2216.
 19. Mulvey, M. R., E. Bryce, D. Boyd, M. Ofner-Agostini, S. Christianson, A. E. Simor, and S. Paton. 2004. Ambler class A extended-spectrum β -lactamase-producing *Escherichia coli* and *Klebsiella* spp. in Canadian hospitals. *Antimicrob. Agents Chemother.* **48**:1204–1214.
 20. National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M7–A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
 21. National Committee for Clinical Laboratory Standards. 2003. Performance standards for antimicrobial susceptibility testing. Thirteenth informational supplement. M100–S13. National Committee for Clinical Laboratory Standards, Wayne, Pa.
 22. Niederhauser, C., L. Kaempf, and I. Heinzer. 2000. Use of the ligase detection reaction-polymerase chain reaction to identify point mutations in extended-spectrum β -lactamases. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**:477–480.
 23. Randegger, C. C., and H. Hächler. 2001. Real-time PCR and melting curve analysis for reliable and rapid detection of SHV extended-spectrum β -lactamases. *Antimicrob. Agents Chemother.* **45**:1730–1736.
 24. Rasheed, J. K., C. Jay, B. Metchock, F. Berkowitz, L. Weigel, J. Crellin, C. Steward, B. Hill, A. A. Medeiros, and F. C. Tenover. 1997. Evolution of extended-spectrum β -lactam resistance (SHV-8) in a strain of *Escherichia coli* during multiple episodes of bacteremia. *Antimicrob. Agents Chemother.* **41**:647–653.
 25. Siu, L. K., P. L. Lu, P. R. Hsueh, F. M. Lin, S. C. Chang, K. T. Luh, M. Ho, and C. Y. Lee. 1999. Bacteremia due to extended-spectrum β -lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in a pediatric oncology ward: clinical features and identification of different plasmids carrying both SHV-5 and TEM-1 genes. *J. Clin. Microbiol.* **37**:4020–4027.
 26. Tham, T. N., C. Mabilat, P. Courvalin, and J. L. Guesdon. 1990. Biotinylated oligonucleotide probes for the detection and the characterization of TEM-type extended broad spectrum β -lactamases in *Enterobacteriaceae*. *FEMS Microbiol. Lett.* **57**:109–115.
 27. Wu, T. L., J. H. Chia, L. H. Su, A. J. Kuo, C. Chu, and C. H. Chiu. 2003. Dissemination of extended-spectrum β -lactamase-producing *Enterobacteriaceae* in pediatric intensive care units. *J. Clin. Microbiol.* **41**:4836–4838.
 28. Yan, J. J., W. C. Ko, S. H. Tsai, H. M. Wu, Y. T. Jin, and J. J. Wu. 2000. Dissemination of CTX-M-3 and CMY-2 β -lactamases among clinical isolates of *Escherichia coli* in southern Taiwan. *J. Clin. Microbiol.* **38**:4320–4325.
 29. Yan, J. J., W. C. Ko, J. J. Wu, S. H. Tsai, and C. L. Chuang. 2004. Epidemiological investigation of bloodstream infections by extended spectrum cephalosporin-resistant *Escherichia coli* in a Taiwanese teaching hospital. *J. Clin. Microbiol.* **42**:3329–3332.
 30. Yan, J. J., S. M. Wu, S. H. Tsai, J. J. Wu, and I. J. Su. 2000. Prevalence of SHV-12 among clinical isolates of *Klebsiella pneumoniae* producing extended-spectrum β -lactamases and identification of a novel AmpC enzyme (CMY-8) in southern Taiwan. *Antimicrob. Agents Chemother.* **44**:1438–1442.
 31. Yang, Y., N. Bhachech, P. A. Bradford, B. D. Jett, D. F. Sahm, and K. Bush. 1998. Ceftazidime-resistant *Klebsiella pneumoniae* and *Escherichia coli* isolates producing TEM-10 and TEM-43 β -lactamases from St. Louis, Missouri. *Antimicrob. Agents Chemother.* **42**:1671–1676.
 32. Yu, W. L., P. L. Winokur, D. L. Von Stein, M. A. Pfaller, J. H. Wang, and R. N. Jones. 2002. First description of *Klebsiella pneumoniae* harboring CTX-M β -lactamases (CTX-M-14 and CTX-M-3) in Taiwan. *Antimicrob. Agents Chemother.* **46**:1098–1100.