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NOTE

Rapid Detection of Extended Spectrum β-Lactamase (ESBL) for Enterobacteriaceae by use of a Multiplex PCR-based Method

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A multiplex PCR method has been developed to classify extended spectrum β-lactamase (ESBL) and plasmid-mediated AmpC β-lactamase (PABL). This method consists of the use of two four-multiplex PCRs for the detection of TEM, OXA, SHV, CTX-M, CMY, and DHA type β-lactamases. We have compared findings from the use of conventional detection methods with that of this newly developed typing method. In testing for 73 ESBL-producing and PABL-producing isolates, 100% of the isolates were correctly identified as previously characterized types and, 44 types of β-lactamases were additionally identified from 33 isolates. This assay not only reduces the time for classification but also increases the accuracy for detection.

Key Words : Extended Spectrum-β Lactamase (ESBL), Rapid classification, Multiplex PCR

Extended spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases (PABLs) hydrolyze a variety of cephalosporins (1-4). Recently, resistance to cephalosporins has become widespread throughout the world, and numerous types of ESBLs and PABLs have been detected in various bacterial organisms (5). Detection and classification of ESBLs and PABLs are important in makingfor clinical decisions regardingfor appropriate therapy and infection control. However, detection and classification are time-consuming and complicated processes, and some types of ESBLs and PABLs are frequently not detected during the detection process (1).

In this study, we described a multiplex PCR method to classify common ESBLs and PABLs and we evaluated the efficiency and accuracy of this method as compared to the conventional method.

Seventy-three (3 *Enterobacter cloacae*, 12 *Escherichia coli*, 21 *Shigella sonnei*, and 37 *Klebsiella pneumoniae* isolates) ESBL-producing and PABL-producing isolates obtained from the Korea Centers for Disease Control and Seoul National University Children's Hospital collection were used in this study (6, 7). All isolates were previously typed for β-lactamase according to usinge the of a conventional method.

Suitable primers for two multiplex PCR reactions, each targeting four different regions, were designed using Primer Express v2.0 (Applied Biosystems, Foster City, CA, USA), which and were synthesized by Bioneer co. (Daejeon, Korea). The first multiplex assay (named Set I) was designed to detect TEM, SHV, CTX-M IV group (8-10), and OXA β-lactamase encoding genes, and the second assay (named Set II) was designedto detect CTX-M I group, CTX-M II group, CMY II, and DHA encoding genes (http://www.

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lahey.org/studies/webt.asp) (Table 1). Both PCR reactions were performed under identical conditions and optimal results were obtained using TaKaRa Ex Taq polymerase (Takara, Tokyo, Japan). Reactions were performed in a final volume of 25 μL containing 5 μL of template DNA, 1× reaction buffer, 0.2 mM of each deoxynucleoside triphosphate, 20 pM of each primer, and 3.5 units of Taq polymerase. Both assays used identical cycling conditions. Reactions were performed in a Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) under the following conditions: denaturation at 94℃ for 5 min followed by 30 cycles of 94℃ for 1 min, 61℃ for 1 min and 72℃ for 1 min, and a final extension of 72℃ for 5 min. After PCR amplification, 3 μL of each reaction was separated by electrophoresis 2% agarose gel (NuSieve 3:1, FMC Bioproducts, Rockland, NY, USA). Both assay products were electrophoresed for 30 min at 100 V in 0.5× TBE buffer. DNA was stained with ethidium bromide (1 μg/mL) and the gels were imaged under UV light. PCR amplicon sizeswere calculated by a comparingson with to molecular weight size markers.

Table 1. The Primers Used to Create in this Study

Assay	Primers	Primer sequence 5' →3'	Size (bp)
Set I	CTX-M IV F	GACAAAGAGAGTGCAACGGATG	501
	CTX-M IV R	TCAGTGCGATCCAGACGAAA	
	TEM F	AGTGCTGCCATAACCATGAGTG	431
	TEM R	CTGACTCCCC GTCGTGTAGATA	
	OXA F	ATTATCTACAGCAGCGCCAGTG	296
	OXA R	TGCATCCACGTCTTTGGTG	
	SHV F	GATGAACGCTTTCCCATGATG	214
	SHV R	CGCTGTTATCGCTCATGGTAA	
Set II	CMY II F	AGCGATCCGGTCACGAAATA	695
	CMY II R	CCCGTTTTATG CACCCATGA	
	CTX M I F	TCCAGAATAAGGAATCCCATGG	621
	CTX M I R	TGCTTTACCCAGCGTCAGAT	
	CTX M II F	ACCGCCGATAATTCGCAGAT	588
	CTX M II R	GATATCGTTGGTGGTGCCATAA	
	DHA F	GTGGTGGACAGCACCATTAAA	314
	DHA R	CCTGCGGTATAGGTAGCCAGAT	

We optimized the conditions of both multiplex PCR reactions to ensure that each amplicon was of the correct size and that a sufficient quantity of product was generated to permit easy detection after gel electrophoresis (Fig. 1). All of the Set I and Set II assays produced single or multiplexed products of the predicted sizes (Fig. 1). After optimization, the assay method was tested on the 73 isolates that produced ESBLs, PABLs or both, where the isolates had already been confirmed by the use of conventional methods. Of the previously known ESBLs and PABLs, those produced by each of the 73 tested isolates were successfully identified with the use of the multiplex assays. We identified additional *bla* genes from 33 isolates (3 *E. cloacae*, 4 *E. coli*, 9 *S. sonnei*, and 17 *K. pneumoniae*) that had not been previously identified

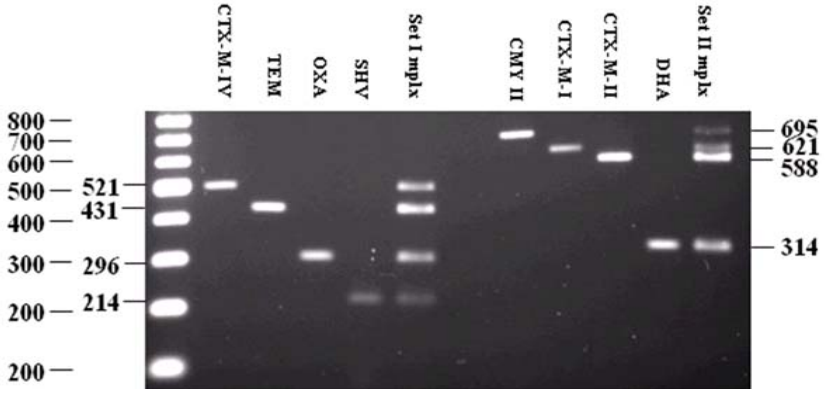

Figure 1. Amplification profiles of each primer set for the Set I and Set II multiplex assays are shown. Set I assay products are shown in the first five lanes with the primer pairs indicated above each lane. (Lane 6 was empty.) Set II assay products are shown in the next five lanes with the primer pairs indicated above each lane. The sizes of the DNA standards are indicated (in base pairs) to the left of the gel image. Amplicon sizes for the Set I reactions are indicated on the left side of the gel, while amplicon sizes for the Set IIreactions are indicated on the right side of the gel.

with the use of conventional methods (Table 2). The additionally identified β-lactamase-encoding genes in the Set I assay were 14 SHV, 8 OXA, 6 TEM and 4 CTX-M-IV types. These were identified from 2 *E. cloacae*, 4 *E. coli*, 3 *S. sonnei*, and 17 *K. pneumoniae* isolates. In four of the *K. pneumoniae* isolates, two additional *bla* genes were identified from each isolate and three additional *bla* genes were identified from E. coli 02-542. Because the SHV-1-like enzymes are ubiquitous in *K. pneumoniae*, detections of these enzymes in *K. pneumoniae* are expected (11). For the Set II assay, additional types of *bla* genes were identified from 12 isolates: 1 *E. cloacae*, 1 *E. coli*, 8 *Shigella* spp., and 2 *K. pneumoniae* isolates. The additional types identified by the Set II assay were 1 CTX-M-1 group, 9 CMY II, and 2 DHA types. The CTX-M-I encoding gene amplified from *S. sonnei* 99-1505 was further identified as $bla_{CTX-M-15}$ by DNA sequencing, and this was the first $bla_{CTX-M-15}$ subtype identified from *Shigella* spp. isolated in Korea.

Classification of ESBL and PABL using phenotypic methods is very difficult since, as many bacterial pathogens are resistant to third-generation cephalosporins, produce two or more ESBLs and/or PABLs, or

Table 2. Comparison of 73 ESBL-producing or PABL-producing *Enterobacteriaceae* using Conventional Method or Multiplex PCR

No.	Species	Reported β-lactamase types	Type detected by multiplex PCR	
			Set I	Set II
04-163	*E. cloacae*	CP*	SHV	
05-172	*E. cloacae*	SHV-2a		CMY I
05-199	*E. cloacae*	CP	SHV	
01-018	*E. coli*	TEM-1, 52	OXA	
02-211	*E. coli*	CTX-M-14, SHV-2a	TEM	
02-542	*E. coli*	CMY-1	CTX-M-IV, OXA, TEM,	
04-128	*E. coli*	CTX-M-14	OXA	CMY II
99-1505	*S. sonnei*	TEM-19	OXA	CTX-M-I
99-2497	*S. sonnei*	TEM-15		CMY II
99-2733	*S. sonnei*	TEM-52		CMY II
99-3093	*S. sonnei*	TEM-15		CMY II
99-3095	*S. sonnei*	TEM-15		CMY II
00-207	*S. sonnei*	TEM-17, 20, 52		CMY II
00-1989	*S. sonnei*	CTX-M-14, TEM-52		CMY II
00-2028	*S. sonnei*	CTX-M-14	TEM	CMY II
00-2852	*S. sonnei*	TEM-15	CTX-M-IV	
01-013	*K. pneumoniae*	CP	TEM	
01-038	*K. pneumoniae*	TEM-52	OXA, SHV	DHA
01-120	*K. pneumoniae*	TEM-52	SHV	
01-123	*K. pneumoniae*	TEM-1, CMY-1	SHV	
01-162	*K. pneumoniae*	SHV-12, CMY-1	OXA	
01-197	*K. pneumoniae*	CTX-M-14, TEM-1	SHV	
02-199	*K. pneumoniae*	CTX-M-14, TEM-1	SHV	
02-219	*K. pneumoniae*	CTX-M-14, TEM-1	SHV	
02-475	*K. pneumoniae*	SHV-12, CMY-1	OXA	
02-514	*K. pneumoniae*	CTX-M-14, TEM-1	SHV	
02-581	*K. pneumoniae*	SHV-12, TEM-1	CTX-M-IV	
02-696	*K. pneumoniae*	TEM-52, TEM-1, DHA-1	CTX-M-IV, SHV	
03-127	*K. pneumoniae*	TEM-1, TEM-52	SHV	
03-167	*K. pneumoniae*	TEM-1, TEM-52	SHV	
03-186	*K. pneumoniae*	CMY-1, SHV-12	OXA, TEM	
04-083	*K. pneumoniae*	TEM, DHA-1	SHV	
05-224	*K. pneumoniae*	CP	SHV, TEM	DHA

*CP : confirmed by phenotype only
†Bold-written types are additional types detected by use of the newly developed multiplex PCR method in this study

have overlapping phenotypes. Therefore, isoelectric focusing (IEF) and type-specific PCR have been employed to classify ESBLs and PABLs. IEF is usually performed first, and the type deduced from the use of IEF is confirmed by type-specific PCR. However, as many of the isoelectric points of the various β-lactamases overlap (1, 8), IEF has only limited detection ability. Therefore, the combination of IEF and type-specific PCR can prevent detection of some ESBLs and PABLs that are present in extended spectrum cephalosporin-resistant bacteria, and furthermore, this combination method is time-consuming.

The multiplex PCR assay developed in this study not only simplifies the detection of ESBLs and PABLs, but also increases the detection ability. This assay takes only 4-5 hours to perform and with the use of this assay, we identified β-lactamases from 33 isolates among the 73 ESBL-producing or PABL-producing isolates where the ESBL and PABL types had already been confirmed by use of conventional methods. Seven additional types were identified, and as there was no overemphasis of one or a few types. We believe that we have developed a fast and accurate assay that will be useful in infectious disease control and prevention.

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