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Multiplex PCR for Simultaneous Detection of Aminoglycoside Resistance Genes in *Escherichia coli* and *Klebsiella pneumoniae*

Hyun Chul Kim, Ji-Hyun Jang, Hyogyeong Kim, Young-Jin Kim, Kyoung-Ryul Lee, and Yun-Tae Kim

Department of Molecular Biology, Seoul Medical Science Institute/Seoul Clinical Laboratories, Seoul 152-766, Korea

The purpose of this study was to develop a multiplex PCR for the detection of *aac(6´)-Ib*, *aph(3´)-Ia*, and *ant(2´´)-Ia*; the genes that encode the most clinically relevant aminoglycoside modifying enzymes (AMEs) in Gram-negative bacteria. Clinical isolates of 80 *E. coli* and 23 *K. pneumoniae* from tertiary university hospital were tested by multiplex PCR. The most prevalent AME gene was *aac(6´)-Ib* which was found in 22.3% of the isolates. Of the total 80 *E. coli* isolates, 1 isolate was found to contain both *aph(3´)-Ia* and *ant(2´´)-Ia* simultaneouly. Of the total 23 *K. pneumoniae* isolates, 2 isolates were found to contain both *aac(6´)-Ib* and *aph(3´)-Ia*, and 1 isolate was found to contain both *aac(6´)-Ib* and *ant(2´´)-Ia* simultaneously. Annual (2005~2009) analysis of isolates that contain the AME genes were of no correlation. The sensitivity and specificity of multiplex PCR in detecting AME genes was 94.4% (34 of 36 cases) and 100%, respectively. We suggest the multiplex PCR method we developed could be highly sensitive and specific in detecting the AME genes of *E. coli* and *K. pneumoniae*. This study could be the first published investigation in which the multiplex PCR method detects *aac(6´)-Ib*, *aph(3´)-Ia*, and *ant(2´´)-Ia* genes.

Keywords : Aminoglycoside, Multiplex PCR, AME genes, *E. coli*, *K. pneumoniae*

INTRODUCTION

Antibiotics are heavily used in medicine and agriculture. To prevent the rising rates of antibiotic resistance, there is a push to establish a legislation to limit the use of antimicrobials for growth promotion and disease prevention in agriculture(Becker, 2010; Spellberg *et al*, 2011). Therefore, it is important to investigate the mechanisms responsible for the persistence of antimicrobial resistance and to determine the potential effects of banning individual antimicrobial agents in food animals versus applying general limitations(Travis *et al*, 2006). In particular, the aminoglycoside antibiotics include many clinically important drugs such as, gentamicin, amikacin, tobramycin, and streptomycin, which are extensively used in the treatment of infections caused by many types of bacteria. These antibiotics constitute a large family of amino-compounds that exhibit broad antibacterial and antiprotozoal activity and have found clinical use since their discovery in the mid-1940s.

Aminoglycoside resistance arises via enzymatic modification of the aminoglycosides, impermeability and multidrug-active efflux systems(Bryan, 1984; Miller *et al.*, 1997; Poole, 2005). Among these, the inactivation of drugs by

Corresponding Author : Kim, Yun-Tae, Department of Molecular Biology, Seoul Medical Science Institute/Seoul Clinical Laboratories, Seoul 152-766, Korea.
Tel: (02)7115-8692 E-mail: ytkim@scllab.co.kr

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plasmid-, or chromosome-encoded AMEs, is the main mechanism of resistance(Poole, 2005). There are three types of AMEs(Shaw *et al.*, 1993; Davies and Wright, 1997; Wright and Thompson, 1999): *N*-Acetyltransferases(AAC, catalyzes acetyl CoA-dependent acetylation of an amino group), *O*-Phosphotransferases(APH, catalyzes ATP-dependent phosphorylationo fahydroxyl group), and *O*-Adenyltransferases(ANT, catalyzes ATP-dependent adenylationofahydroxyl group). The production of AMEs, including AAC(3)-I, AAC(6′)-I, APH(3′)-I, APH(3′)-VI, and ANT(2″)-I occurs mainly in Gram-negative pathogens(Lee *et al.*, 1987; Gaynes *et al.*, 1988; Reshed'ko, 2004). Unique antibiotics resistance profiles and protein designations write the Roman numbers and letters. The AAC(3)-I is classified by resistance to fortimicin, and gentamicin. Two aac(3)-I genes have been determined (*aac(3)-Ia*, and *aac(3)-Ib*). The AAC(6′)-I is classified by resistance to amikacin, tobramycin, dibekacin, 2′-N-ethylnetilmicin, 5-episisomicin, netilmicin, and sisomicin. Eight *aac(6′)-I* genes have been determined(*aac(6′)-Ia*, *aac(6′)-Ib*, *aac(6′)-Ic*, *aac(6′)-Id*, *aac(6′)-Ie*, *aac(6′)-If*, *aac(6′)-Ig*, *aac(6′)-Ih*, and *aac(6′)-Ii*). The APH(3′)-I is classified by resistance to neomycin, kanamycin, paromomycin, lividomycin, ribostamycin, and gentamicin B. Three *aph(3′)-I* genes have been determined(*aph(3′)-Ia*, *aph(3′)-Ib*, and *aph(3′)-I*). The APH(3′)-VI is classified by resistance to kanamycin, paromomycin, neomycin, butirosin, ribostamycin, and gentamicin B, as well as amikacin and isepamicin. Two *aph(3′)-I* genes have been determined(*aph(3′)-Ia*, and *aph(3′)-Ib*). The ANT(2″)-I is classified by resistance to tobramycin, kanamycin, gentamicin, dibekacin, and sisomicin. Two *ant(3″)-I* genes have been determined(*ant(3″)-Ia*, and *ant(3″)-Ib*)(Shaw *et al.*, 1993).

Previous studies have shown that over 50 different AMEs have been identified(Davies and Wright, 1997). Enzymatic modification results in a high level of antibiotics resistance (Kucers *et al.*, 1997). Most enzyme-related resistance in Gram-negative bacilli is due to multiple genes. Mandell *et al.* hypothesized that the enzymes are derived from organisms that make the aminoglycoside or from the mutation of genes that encode the enzymes involved in cellular respiration(Gilbert, 2000). Resistance genes are often carried on mobile genetic elements, such as phages, transposons, and plasmids, and are subject to frequent horizontal transfers.

Researchers have tried different approaches to detect these resistance genes. Polymerase chain reaction(PCR) appear to be the more rapid, sensitive, and specific assay for such detection compared to southern blot hybridization, macrorestriction, fingerprinting, and determining the minimal inhibitory concentration(MIC)(Vanhoof *et al.*, 1994). In particular, multiplex PCR that detects several genes simultaneously in the same PCR tube has the advantage of identifying genotypic resistance for several antibiotics more rapidly and reliably(Geha *et al.*, 1994; Martineau, 2000).

In this report, we describe a rapid multiplex PCR assay for the simultaneous detection of genes encoding AMEs and evaluate the prevalence of these resistance genes in *E. coli* and *K. pneumoniae* isolates in a single reaction. The objective of this study was to develop a rapid multiplex PCR assay for the simultaneous detection of genes encoding AMEs and to evaluate the prevalence of these resistance genes in *E. coli* and *K. pneumoniae* isolates in a single reaction.

MATERIALS AND METHODS

Bacterial strains

One hundred three Gram-negative bacteria from human blood sources were evaluated for three aminoglycoside resistance genes using multiplex PCR. Between July 2005 and April 2009, we collected the 103 strains(*E. coli* 80,

K. pneumoniae 23) which can produce enzyme(ESBL, Extended spectrum beta lactamase) and were the intermediate to high resistant to gentamicin and ciprofloxacin simultaneously from clinical isolates. Due to the high resistant to gentamicin, the strains were suspected of aminoglycoside resistance strain. Clinical isolates were separate patients from tertiary university hospital in the Republic of Korea. *E. coli* and *K. pneumoniae* strains were identified using Vitek2 system(BioMerieux, Marcy l'Etoile, France) and 16S rRNA sequencing. Stock cultures were stored frozen(−70℃) in Luria Bertani(LB), containing 15% glycerol.

DNA purification from culture samples

Using a QIAamp DNA mini kit(QIAGEN, Hilden, Germany), DNA was purified from *E. coli* and *K. pneumoniae* isolates. A single colony of the isolates was inoculated into LB and was incubated for 16hrs at 37℃. The DNA was then extracted according to the manufacturer's instructions.

Primers for single PCR and Multiplex PCR

Using Primer3Plus program(Untergasser *et al.*, 2007) and ClustalX program, three primer sets specific for *aac(6´)−Ib* (main GenBank access code M21682), *aph(3´)−Ia*(main GenBank access code HQ380034), and *ant(2´´)−Ia*(main GenBank access code X12618) gene were designed within the nucleotide sequence of the published region of each resistance gene(Table 1).

PCR experiments were performed in a volume of 20 ul with the following in a reaction tube: DNA template(obtained as described above), 0.5 mM(each) deoxynucleotide triphosphate, 10X PCR buffer, 0.25 U/ul polymerase, and primer sets for the three aminoglycoside resistance genes(total 6 primers; see Table 1). All primers used in this study were provided by the Bioneer(Oligo, Bioneer, Daejeon, Korea). The amount of each primer for single PCR and multiplex PCR was 10.0 pmol and 0.4 pmol, respectively. Table 2 shows single PCR conditions. Multiplex PCR was initial denaturation of 12 min at 95℃; 35 cycles of 50 sec at 95℃, 50 sec at 51℃, and 1 min at 72℃; and a final extension step of 5 min at 72℃. After amplification, 6 ul aliquot of each amplication product was analyzed using electrophoresis on 1% agarose gels cast and ran in 0.5X TBE buffer. Gel was stained with ethidium bromide and visualized using transmitted ultraviolet illumination and photographed using gel documentation system. DNA from two strains(*E. coli* and *K. pneumoniae*) that together contained the three AME genes of interest were placed in a single tube for each set of multiplex PCR experiments, and the resulting three PCR products were used as positive controls and size marker(100bp marker, Cosmogenetech, Seoul, Korea) in gel electrophoresis.

In preliminary experiments, the aminoglycoside resistance gene content of all 103 isolates had been determined using single PCR methods with one pair of primer per re-

Table 1. Nucleotide sequences of primer sets used to amplify aminoglycoside resistance genes in multiplex PCR experiments

Aminoglycoside resistance gene	Primer sequence	Product size (bp)	Ref.
aac(6´)−Ib	5´−AGTACTTGCCAAGCGTTTTAGCGC−3´	365	This study
	5´−CATGTACACGGCTGGACCAT−3´		
aph(3´)−Ia	5´−ATGGGCTCGCGATAATGTCG−3´	734	This study
	5´−AGAAAAACTCATCGAGCATC−3´		Chen *et al.*, 2010
ant(2´´)−Ia	5´−ATGCAAGTAGCGTATGCGCT−3´	477	This study
	5´−TCCCCGATCTCCGCTAAGAA−3´		This study

Table 2. PCR conditions for single PCR

Aminoglycoside resistance gene	PCR condition		
aac(6´)–Ib	1 cycle	32 cycles	1 cycle
	95°C, 12 min	95°C, 40 sec; 58°C, 40 sec; 72°C, 40 sec	72°C, 5 min
aph(3´)–Ia	1 cycle	32 cycles	1 cycle
	95°C, 12 min	95°C, 40 sec; 57°C, 40 sec; 72°C, 1 min	72°C, 5 min
ant(2´´)–Ia	1 cycle	32 cycles	1 cycle
	95°C, 12 min	95°C, 40 sec; 57°C, 40 sec; 72°C, 50 sec	72°C, 5 min

action for each of three aminoglycoside resistance genes (*aac(6´)–Ib*, *aph(3´)–Ia*, and *ant(2´´)–Ia*). Also, the primer concentration and the annealing temperature were optimized.

DNA sequencing and analysis

The PCR products were sequenced twice with an ABI 3130XL DNA genetic analysis automated sequencer according to manufacturer's instructions(3130XL DNA genetic analyzer, Applied Biosystems, Foster City, USA). To eliminate errors caused by amplification artifacts, the forward and reverse sequences of each AME genes sequence were determined for products from at least two independent PCR. The sequences of the PCR products were compared with known AME genes sequence in NCBI web site.

Result

We studied three other aminoglycoside resistance genes that encode AMEs found in Gram–negative bacteria: *aac(6´)–Ib*, *aph(3´)–Ia*, and *ant(2´´)–Ia*(Reshed'ko, 2004). We designed three sets of primers that were specific for *aac(6´)–Ib*, *aph(3´)–Ia*, and *ant(2´´)–Ia*(Table 1). To characterize the AME genes detected in *E. coli* and *K. pneumoniae*, the effect of primer concentration(4.0~0.04 pmol) and annealing temperature(49.0~55.0℃) were examined using multiplex PCR(Elnifro et al., 2000). The optimal primer concentration and annealing temperature for multiplex PCR detection of AME genes was found to be 0.4 pmol(each primer) and 51.0℃, respectively(Fig. 1). Amplified DNA fragments of three different sizes(365, 477, and 734 bp) were detected in a representative agarose gel electrophoresis(Fig. 2). A positive control(DNA) from

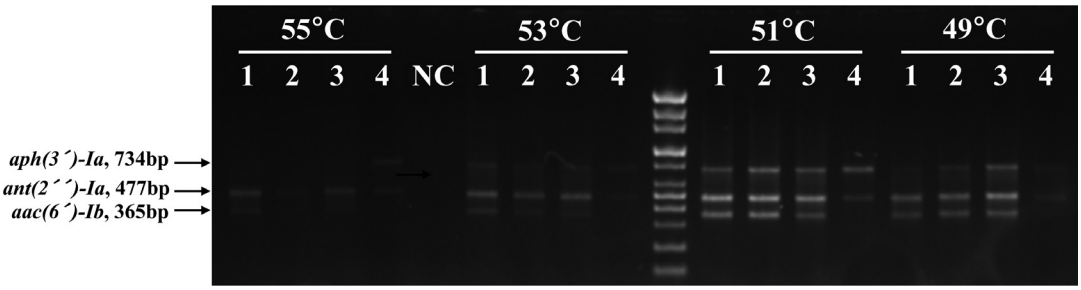

Fig. 1. The optimal primer concentration and annealing temperature for multiplex PCR detection of AME genes. Lanes: 1, 4.0 pmol; 2, 0.4 pmol; 3, 0.1 pmol; 4, 0.04 pmol.

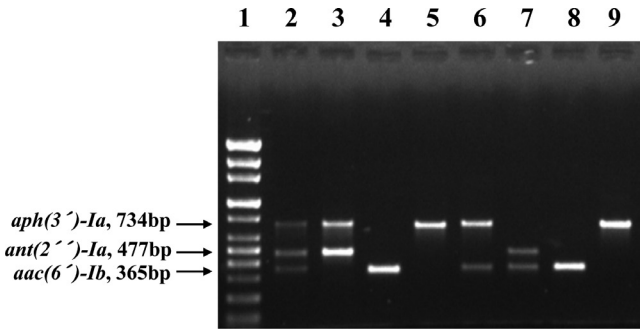

Fig. 2. Representative patterns of DNA fragments amplified using multiplex PCR from *E. coli* and *K. pneumoniae*. Lanes: 1, 100bp DNA ladder; 2, Positive control (DNA) from two clinical isolates that together contained the three AME genes was detected in a single tube for multiplex PCR method; 3, *aph(3´)–Ia* + *ant(2´´)–Ia* from *E. coli* isolate; 4, *aac(6´)–Ib* from *E. coli* isolate; 5, *aph(3´)–Ia* from *E. coli* isolate; 6, *aph(3´)–Ia* + *aac(6´)–Ib* from *K. pneumoniae* isolate; 7, *ant(2´´)–Ia* + *aac(6´)–Ib* from *K. pneumoniae* isolate; 8, *aac(6´)–Ib* from *K. pneumoniae* isolate; 9, *aph(3´)–Ia* from *K. pneumoniae* isolate.

two clinical isolates that together contained the three AME genes was detected in a single tube using the multiplex PCR method. The PCR product patterns of representative strains showed *aph(3´)–Ia* + *ant(2´´)–Ia*, *aac(6´)–Ib*, and *aph(3´)–Ia* genes in *E. coli* and *aph(3´)–Ia* + *aac(6´)–Ib*, *ant(2´´)–Ia* + *aac(6´)–Ib*, *aac(6´)–Ib*, and *aph(3´)–Ia* genes in *K. pneumoniae*, respectively(Fig. 2). Non-specific background amplification products were not detected in this multiplex PCR assay. Therefore, the specificity of the primers selected in this study for the multiplex PCR was proved. Each amplified the DNA fragments were consistently observed comparing positive control in the several strains. We tested AME genes from total clinical 103 isolates using agarose electrophoresis(Fig. 3). We could find the three PCR products of the AME genes, which were *aac(6´)–Ib*, *aph(3´)–Ia*, and *ant(2´´)–Ia*, in the sequence

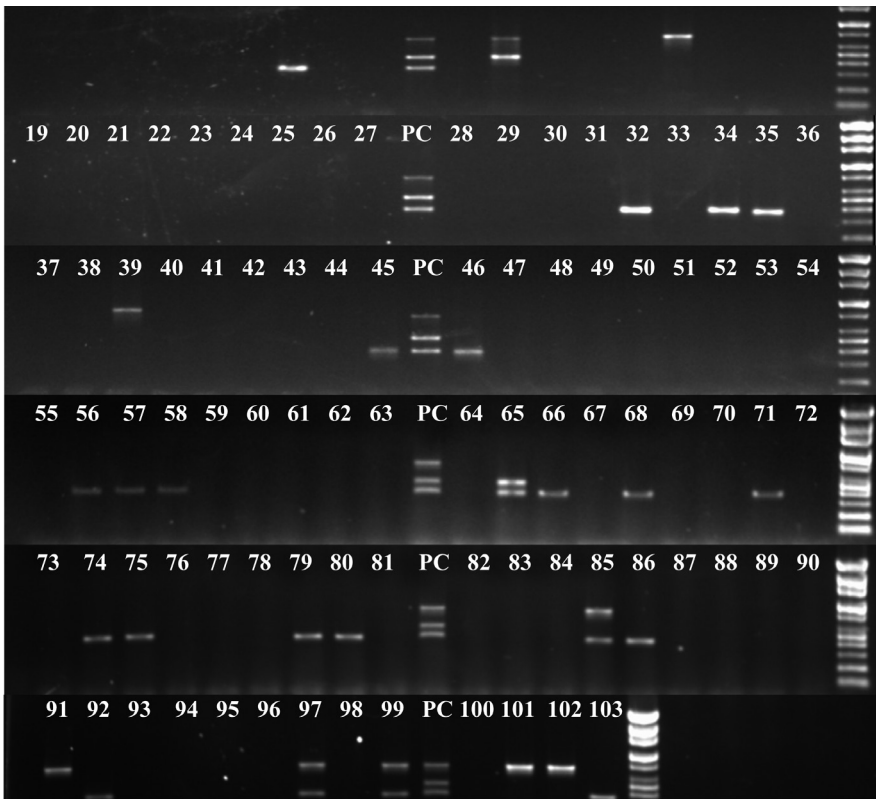

Fig. 3. Agarose gel electrophoresis of DNA fragments amplified using multiplex PCR from 103 isolates. PC (Positive control) from two clinical isolates that together contained the three AME genes was detected in a single tube for multiplex PCR method.

analysis. A similarity search of the AME gene sequence was carried out using the blast program, available at the NCBI BLAST web site.

The most prevalent AME gene in a total of 103 isolates was *aac(6´)–Ib*, found in 23(22.3%; 16 *E. coli*, 7 *K. pneumoniae*) isolates; *aph(3´)–Ia* and *ant(2´´)–Ia* were found in 9(8.7%; 5 *E. coli,* 4 *K. pneumoniae*) isolates and 2(1.9%; 1 *E. coli*, 1 *K. pneumoniae*) isolates, respectively(Fig. 4). Of the total 80 *E. coli* isolates, 1 isolate was found to contain both *aph(3´)–Ia* and *ant(2´´)–Ia* simultaneously. Of the total 23 *K. neumoniae* isolates, 2 isolates were found to contain both *aac(6´)–Ib* and *aph(3´)–Ia*, and 1 isolate was found to contain both *aac(6´)–Ib* and *ant(2´´)–Ia* simultaneously. Seventy–three(70.8%) isolates were not found to have any AME genes(Fig. 4).

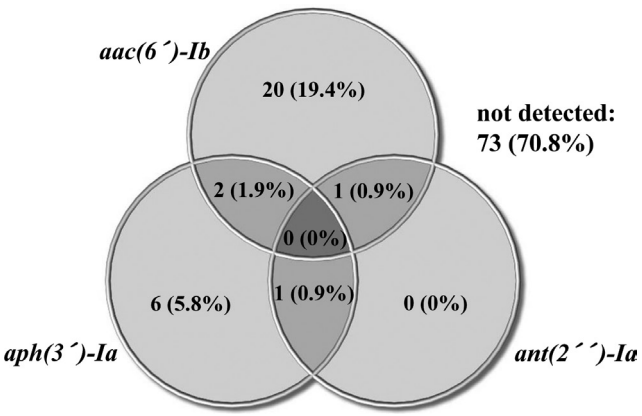

Fig. 4. The incidence of genes encoding aminoglycoside modifying enzyme, as determined by multiplex PCR in 80 *E. coli* and 23 *K. pneumoniae*. The most prevalent AME gene in a total of 103 isolates was *aac(6´)–Ib*, found in 23(22.3%; 16 *E. coli*, 7 *K. pneumoniae*) isolates; *aph(3´)–Ia* and *ant(2´´)–Ia* were found in 9(8.7%; 5 *E. coli*, 4 *K. pneumoniae*) isolates and 2(1.9%; 1 *E. coli*, 1 *K. pneumoniae*) isolates, respectively.

To compare the performance of single PCR and multiplex PCR, the single PCR of AME genes(*aac(6´)–Ib*, *aph(3´)–Ia*, and *ant(2´´)–Ia*) was tested. We could detect the AME genes in all 103 isolates using the multiplex PCR we developed; compared with single PCR, the multiplex PCR had an overall sensitivity of 94.4%(34 of 36 cases) and an overall specificity of 100%(273 of 273 cases)(Table 3). To put it more concretely, compared with single PCR, the sensitivities of multiplex PCR in detecting *aac(6´)–Ib*, *aph(3´)–Ia*, and *ant(2´´)–Ia* were 95.8%(23 of 24 cases), 90.0%(9 of 10 cases), and 100.0%(2 of 2 cases), respectively. The specificities of multiplex PCR in detecting the three AME genes were 100%(Table 3). Also, compared with single PCR, the sensitivities of multiplex PCR in detecting *E. coli* and *K. pneumoniae* were 95.6%(22 of 23 cases) and 92.3%(12 of 13 cases), respectively. The specificities of multiplex PCR for each strain were 100%(data not shown).

Discussion

Aminoglycosides play an important role in serious *E. coli* and *K. pneumoniae* infections, despite reports of increased resistance to drugs. Several reports have stated that aminoglycoside(gentamicin) resistance is closely related to ciprofloxacin resistance(Haller, 1985; Mulder *et al.*, 1997; Mandal *et al.*, 2003; Pépin *et al.*, 2009). Gentamicin was

Table 3. Performance of multiplex PCR compared with single PCR for detecting AME genes

	% Sensitivity		% Specificity	
	Single PCR	Multiplex PCR	Single PCR	Multiplex PCR
aac(6´)–Ib	100 (24/24)	95.8 (23/24)	100 (79/79)	100 (79/79)
aph(3´)–Ia	100 (10/10)	90.0 (9/10)	100 (93/93)	100 (93/93)
ant(2´´)–Ia	100 (2/2)	100 (2/2)	100 (101/101)	100 (101/101)
Total	100 (36/36)	94.4 (34/36)	100 (273/273)	100 (273/273)

the most active against Gram-negative bacteria, including *E. coli* and *K. pneumoniae*, and is often used in combination with either beta-lactam or daptomycin(Leclercq *et al.*, 1991; Moulds and Jeyasingham, 2010). The strains tested in this study had high-level resistance to gentamicin and ciprofloxacin simultaneously.

The AMEs were classified as AAC, APH, and ANT. Many studies have found a correlation between common genes encoding for AMEs and aminoglycoside resistance(Kobayashi *et al.*, 2001; Choi *et al.*, 2003; Vakulenko *et al.*, 2003; Chen *et al.*, 2006). In this study, we investigated AAC(6′)-I, APH(3′)-I, and ANT(2″)-I in Gram-negative bacteria.

The AME genes(*aac(6′)-Ib*, *aph(3′)-Ia*, and *ant(2″)-Ia*) we studied appeared frequently in clinical isolates and correlated with resistance to antibiotics. At least six genes that encode this AAC(6′)-I have been identified in a concrete form: *aac(6′)-Ia*(Tenover *et al.*, 1988) was extremely rare (0.1%); *aac(6′)-Ib*(Tran Van Nhieu and Collatz, 1987; Nobuta *et al.*, 1988) was the most common, found in 70.6% of strains expressing this AAC(6′)-I; *aac(6′)-Ic*(Shaw *et al.*, 1992) was found in 21.3% of AAC(6')-I strains; *aac(6′)-Id*(Schmidt *et al.*, 1988; Shaw *et al.*, 1992) was extremely rare; *aac(6′)-Ie*(Ferretti *et al.*, 1986; Rouch *et al.*, 1987), which encodes the amino-terminal portion of the bifunctional enzyme AAC(6′) + APH(2″), was detected only in Gram-positive bacteria; and *aac(6′)-If* (Teran *et al.*, 1991) was cloned from *Enterobacter cloacae*. In this study, 23(22.3%) isolates of the total 103 isolates had *aac(6′)-Ib*. Among the 23 isolates, 2 isolates were found to contain both *aac(6′)-Ib* and *aph(3′)-Ia*, and 1 isolate was found to contain both *aac(6′)-Ib* and *ant(2″)-Ia* simultaneously.

The precise frequency of APH(3′)-I varied among different species: 46% for Gram-negative bacteria, 6.6% for *Pseudomonas* spp., 27.5% for *Serratia* spp., and 49.5% for *Acinetobacter* spp.(Shaw *et al.*, 1993). Three genes that encode this APH(3′)-I have been cloned: *aph(3′)-Ia* from Tn*903*(Oka *et al.*, 1981); *aph(3′)-Ib* from plasmid RP4(Pansegrau *et al.*, 1987); and *aph(3′)-Ic* from an isolate of *K. pneumoniae* that showed increased resistance to elimination using neomycin(Lee *et al.*, 1991). The most widely distributed among these is encoded by *aph(3′)-Ia*, which is frequently found on transposable elements, e.g., Tn*903*(Oka *et al.*, 1981). The *aph(3′)-Ic* gene is nearly identical to *aph(3′)-Ia*(seven nucleotide substitutions) (Lee *et al.*, 1991), and therefore these strains would hybridize with the *aph(3′)-Ia* probe. However, since the *aph(3′)-Ia* and *aph(3′)-Ib* genes share only 60% DNA homology, it is quite probable that the *aph(3′)-Ia* probe would not hybridize with strains harboring the *aph(3′)-Ib* gene under the strict hybridization conditions used (Shaw *et al.*, 1991). In the current study, 9(8.7%) isolates of the total 103 isolates detected *aph(3′)-Ia*. Of the 9 isolates, 2 isolates were found to contain both *aph(3′)-Ia* and *aac(6′)-Ib*, and 1 isolate was found to contain both *aph(3′)-Ia* and *ant(2″)-Ia* simultaneously.

Also, ANT(2″)-I is widespread among all Gram-negative bacteria and was found in 14.9~21.1% of strains tested. Three genes encoding 2″-*O*-adenylyltransferase activity have been reported(Lee *et al.*, 1987). The a*nt(2″)-Ia* gene is the most frequent. It was observed in 87% of strains expressing ANT(2″)-I, similar to the frequency observed(77.6%) in a previous study by Shaw *et al.*(Shaw *et al.*, 1991). Two(1.9%) isolates of the total 103 isolates detected *ant(2″)-Ia* in our results. Of these, 1 isolate was found to contain both *ant(2″)-Ia* and *aac(6′)-Ib*, and another 1 isolate was found to contain both *ant(2″)-Ia* and *aph(3′)-Ia* simultaneously. Meanwhile, the strain carrying *aac(6′)-Ib*, *aph(3′)-Ia,* and *ant(2″)-Ia* simultaneously was not detected in any isolates in this study. The detection ratio of the AMEs is relatively low in our results compared to previous research because we targeted a small number of strains in this study. Therefore, additional data

for a larger number of clinical isolates will be needed for a more exact investigation.

As shown in Table 4, clinical microorganisms contain the AME genes or records are similar on a year-on-year basis(30% of 2005, 25% of 2006, 33% of 2007, 33% of 2008, and 24% of 2009). In *Staphylococcus aureus*, the *ant(4´)-Ia* was the most frequent gene(58%), and *aac(6´)-Ie/aph(2´´)* and *aph(3´)*-IIIa genes were found in 46% and 6% of the isolates, respectively in the other study(Yadegar *et al.*, 2009). There is a little difference between the results and the results of our study. Because it seems that an objects of this study were Gram negative bacteria(*E. coli*, *K. pneumoniae*).

Drug inactivation by AMEs is the primary mechanism of aminoglycoside resistance. Culture-based methods, including MIC, are generally used to detect AME-related clinical bacteria. However, the mechanisms of aminoglycoside resistance have become more complex with the increased use of aminoglycosides over time. Moreover, there is no rapid and reliable method for detecting aminoglycoside resistance. The detection of the aminoglycoside-resistant gene using a PCR assay is now considered the gold standard. In particular, the multiplex PCR method that simultaneously detects several genes in a single reaction has the advantage of identifying genotypic resistance for several antibiotics more rapidly and reliably. Several studies have used multiplex PCR to detect genes encoding aminoglycoside-resistance and genes for species identification in cases of Gram-negative bacteria infection(Choi *et al.*, 2003).

The optimal conditions for multiplex PCR were established by varying the concentration of primers at different annealing temperatures. The optimal conditions determined were an annealing temperature of 51℃, primer concentration of 0.4 pmol, an MgCl2 concentration of 2.5mM, and dNTP concentration of 2mM. Under these optimal conditions we secure the multiplex PCR fidelity.

Our objective is to develop a multiplex PCR method that can detect *aac(6´)-Ib*, *aph(3´)-Ia*, and *ant(2´´)-Ia*, the most representative AME genes in *E. coli* and *K. pneumoniae* from clinical isolates. The capability of multiplex PCR to detect AME genes in clinical bacteria simultaneously and with high specificity provides a new approach to the diagnosis of antibiotic resistance in clinical specimens. Even though the sensitivity of multiplex PCR is marginally less than single PCR, this method we developed is readily available due to the speed and low cost of multiplex PCR. We successfully developed a rapid multiplex PCR method that was performed in less than 3 hrs. This method is also accurate compared with the conventional method. We

Table 4. Annual rate of isolates cotain the AME genes in korea from 2005 to 2009

Aminoglycoside resistance gene	Percentage of isolates contain AME genes				
	2005	2006	2007	2008	2009
aac(6´)-Ib	20 (4/20)	15 (3/20)	19 (4/21)	24 (5/21)	19 (4/21)
aph(3´)-Ia	5 (1/20)	5 (1/20)	10 (2/21)	5 (1/21)	5 (1/21)
ant(2´´)-Ia	0 (0/20)	0 (0/20)	0 (0/21)	0 (0/21)	0 (0/21)
aac(6´)-Ib + aph(3´)-Ia	5 (1/20)	0 (0/20)	5 (1/21)	0 (0/21)	0 (0/21)
aac(6´)-Ib + ant(2´´)-Ia	0 (0/20)	5 (1/20)	0 (0/21)	0 (0/21)	0 (0/21)
aph(3´)-Ia + ant(2´´)-Ia	0 (0/20)	0 (0/20)	0 (0/21)	5 (1/21)	0 (0/21)
Total	30 (6/20)	25 (5/20)	33 (7/21)	33 7/21)	24 (5/21)

think our results reinforce the availability of DNA-based assays to detect antibiotic-resistant genes associated with Gram-negative clinical bacterial infections. However, additional data for a larger number of clinical isolates will be required before this method can be applied in clinical practice. In view of the high prevalence of aminoglycoside resistance observed in this study, periodic surveillance of aminoglycoside resistance and of the corresponding genes is needed.

We suggest the multiplex PCR method we developed can be highly sensitive and specific in detecting the AME genes of *E. coli* and *K. pneumoniae*. In this study, the multiplex PCR method combined with *aac(6´)-Ib*, *aph(3´)-Ia*, and *ant(2´´)-Ia* gene is the first invention published in Korea.

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