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Identification of strain harboring both *aac(6')-Ib* and *aac(6')-Ib-cr* variant simultaneously in *Escherichia coli* and *Klebsiella pneumoniae*

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The *aac(6')-Ib* gene is the most prevalent gene that encodes aminoglycoside-modifying enzymes and confers resistance to tobramycin, kanamycin, and amikacin. The *aac(6')-Ib-cr* variant gene can induce resistance against aminoglycoside and fluoroquinolone simultaneously. Two main methods, sequence analysis and the restriction enzyme method, can detect the *aac(6')-Ib-cr* variant in clinical strains. We collected the 85 strains that were believed to be *aac(6')-Ib* positive from clinical isolates. Among them, 38 strains were the wild-type; the remaining 47 strains were the *aac(6')-Ib-cr* variant. Of these 47 strains, 19 simultaneously harbored *aac(6')-Ib* and *aac(6')-Ib-cr*. Our study aims to report the characteristics of the 19 strains that simultaneously harbored both genes. This study is the first investigation published in Korea of strains that included both *aac(6')-Ib* and *aac(6')-Ib-cr* variant. [BMB reports 2011; 44(4): 262-266]

INTRODUCTION

Quinolone resistance has been mediated by chromosomal mutations in bacterial topoisomerase genes, genes regulating expression of efflux pumps, or both (1). Recently, a new mechanism of transferable quinolone resistance was reported enzymatic inactivation of certain quinolones. Plasmid-mediated quinolone resistances (PMQR) have been described. They have been reported worldwide in unrelated enterobacterial species and are usually associated with mobile elements (2). *aac(6')-Ib*, the most prevalent aminoglycoside modifying enzyme that confers resistance to tobramycin, kanamycin, and amikacin, was first identified in *Klebsiella pneumoniae* isolates in 1986. Since then several variants of this enzyme have been described (3-5). The cr variant of *aac(6')-Ib* encodes an aminoglycoside acetyltransferase that contributes reduced susceptibility to ciprofloxacin by N-acetylation of its piperazinyl amine (6). The *cr* in *aac(6')-Ib-cr* means ciprofloxacin (fluoroquinolone) resistance. Thus, the *aac(6')-Ib-cr* variant gene can induce resistance against aminoglycoside and fluoroquinolone simultaneously. The gene has been discovered in various locations, including the USA, Canada, Argentina, Portugal, France, China and Spain (7). The *aac(6')-Ib-cr* variant has two amino acid changes, Trp102Arg and Asp179Tyr, which together are necessary and adequate for the enzyme's ability to acetylate ciprofloxacin (6, 8). It is very important to determine the strains, which are harboring *aac(6')-Ib-cr* variant, in order to use fluoroquinolone antimicrobials appropriately for human therapeutics. Because fluoroquinolone are among the three most commonly used antimicrobials in human therapeutics. Interestingly, we found some strains that are harboring both *aac(6')-Ib* and *aac(6')-Ib-cr* variant in the same cell isolated from clinical. We consider that it will be processing transitions from one stage to the next, presenting two genes simultaneously (9). We believe this hypothesis can be explained by theory of the genetic heterozygote. According to the theory of the heterozygote, genetic heterozygote means that different mutations within a single gene locus cause the same phenotypic expression (10). These coexistence of wild-type *aac(6')-Ib* and *aac(6')-Ib-cr* variant (at nucleotide 304 by a T→C) alleles in an isolate was a surprisingly common phenomenon, a survey which was very recently supported in the other study (11). Also, these isolates where a proportion of the cells have acquired a *cr* allele, and under sustained antibiotic exposure these cells would quickly dominate the population (12).

In order to confirm this phenomenon, we have performed various experiments repeatedly, including sequence analysis, restriction enzyme digestion, cloning, and Denaturing High-Performance Liquid Chromatography (DHPLC) analysis. The aim of this study is to report the characteristics of genetic strains harboring both *aac(6')-Ib* and the *aac(6')-Ib-cr* variant si-

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Table 1. Correlation between nucleotide changes and the number of the fragments of DNA when PCR products of *aac(6')-Ib* is treated by restriction enzyme *Bts*CI and MIC of amikacin and ciprofloxacin in the strains

Changes at 304 nucleotide of 102 codon	The number of the fragments of DNA on the gel electrophoresis			Resistance patterns			
				Amikacin		Ciprofloxacin	
	One fragment (482 bp)	Two fragments (272 bp, 210 bp)	Three fragments (482 bp, 272 bp, 210 bp)	R %§	MIC‖	R %	MIC
GGATGG* *aac(6')-Ib*		38		28.9% (11/38)	22.11	92.1% (35/38)	13.12
GGACGG† *aac(6')-Ib-cr*	28			28.6% (8/28)	22.6	85.7% (24/28)	14.47
GGA(T/C)GG‡ *aac(6')-Ib-cr*	1		18	21.1% (4/19)	22.11	78.9% (15/19)	11.76

*The strains, which harbor *aac(6')-Ib* gene that possess amino acid Trp (TGG) in 102 codon, were showed two fragments (272 bp, 210 bp) of DNA on the gel electrophoresis, †The strains, which harbor *aac(6')-Ib-cr* gene that possess amino acid Arg (CGG) in 102 codon, were showed one fragment (482 bp) of DNA on the gel electrophoresis, ‡The strains, which are having T (base) and C (base) simultaneously at 304 nucleotide of 102 codon, were showed three fragment (482 bp, 272 bp, 210 bp) of DNA on the gel electrophoresis, §Percent resistant, ‖Mean value of minimum inhibitory concentration (ug/ml)

multaneously in the same cell. This study, the first of its type published in Korea, will be useful in the further study of genes that confer resistance to antibiotics.

RESULTS AND DISCUSSION

Thirty-eight of the 85 strains were the wild-type, *aac(6')-Ib*; the remaining 47 strains were the *aac(6')-Ib-cr* variant. Among these *aac(6')-Ib-cr* variant strains, 28 strains were typical variants and possessed amino acid Arg (CGG) in 102 codon. However, the remaining 19 strains had both T (base) and C (base) simultaneously at nucleotide position 304 (102 codon) (Table 1, Fig. 1A). The 47 *aac(6')-Ib-cr* variant strains also demonstrated changes in the amino acid at the 179 codon (Asp→179Tyr). Interestingly, 17 of the 19 strains with both T and C at nucleotide position 304 (102 codon) also had both G and T simultaneously at nucleotide position 535 (179 codon); we believe this to be evidence of genetic heterogeneity.

All polymerase chain reaction (PCR) products positive for *aac(6')-Ib* were further analyzed by digestion with *Bts*CI (Fig. 1B) and by direct sequencing to identify *aac(6')-Ib-cr*, which lacks the *Bts*CI restriction site present in the wild-type gene (13). As a results, 38 strains were the *aac(6')-Ib* genes, which possess amino acid Trp (TGG) in 102 codon (Fig. 1A(a)). The wild-type *aac(6')-Ib* showed two fragments (272 bp, 210 bp) of DNA on gel electrophoresis after treatment with restriction enzyme *Bts*CI (Fig. 1B). The 28 strains that harbor *aac(6')-Ib-cr* gene and possess amino acid Arg (CGG) in 102 codon (Fig. 1A(b)), showed one fragment (482 bp) of DNA on gel electrophoresis when treated by restriction enzyme *Bts*CI; this is attributable to the lack of a recognition site on the *aac(6')-Ib-cr* gene variant (the site is cut by *Bts*CI; Fig. 1B).

Interestingly, 18 of the 19 strains harboring both T and C simultaneously at nucleotide position 304 (Fig. 1A(c)) showed 3 fragments (482 bp, 272 bp, 210 bp) of DNA on gel electrophoresis when treated by restriction enzyme *Bts*CI (Fig. 1B). To

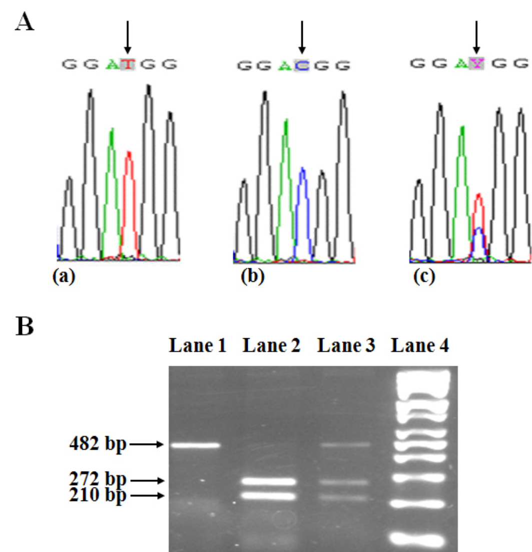

Fig. 1. (A) Direct sequencing chromatographs of the *aac(6´)-Ib* and *aac(6´)-Ib-cr* are shown. (a) is indicated the wild-type *aac(6´)-Ib* which possess amino acid Trp (TGG) in 102 codon. (b) is indicated the typical *aac(6´)-Ib-cr* variants which possess amino acid Arg (CGG) in 102 codon. (c) is indicated the strain that are having both T and C simultaneously at nucleotide position 304 of the 102 codon. The strains are harboring *aac(6´)-Ib* and *aac(6´)-Ib-cr* variants simultaneously. Three arrows indicate sequence at nucleotide position 304 of the 102 codon, respectively. (B) *Bts*CI digestion of purified *aac(6´)-Ib* PCR products. Lane 1, The PCR products which were not cut by *Bts*CI digestion (*aac(6´)-Ib-cr* gene); lane 2, The PCR products which were cut by *Bts*CI digestion (*aac(6´)-Ib* gene); lane 3, The PCR products which showed three fragments by *Bts*CI digestion (*aac(6´)-Ib-cr* gene); lane 4, DNA ladder (100 bp). When the positive *aac(6´)-Ib* PCR products were purified and treated with *Bts*CI, only those of wild-type gene were digested into two fragments product (lane 2) while those of the cr variant were not cut (lane 1) and the strains, which are having both T and C simultaneously at nucleotide position 304 of the 102 codon, were digested into three fragments product (lane 3).

demonstrate this phenomenon again, these genes were cloned into DH5α using pGEM® T-easy vector sequence. Cloning was also performed using polymerase chain reaction (PCR) and direct sequencing; these methods yielded results that were the same as those for the insert genes that are cloned before. These cloning methods allowed description of the genetic types that were simultaneously harboring both *aac(6')-Ib* and the *aac(6')-Ib-cr* variant. In other words, *aac(6')-Ib-cr* variants manifested as two distinct types (at nucleotide position 304 by a T→C or T→T/C) during this study. In the first type, that T converts into C were appeared one fragment of DNA (482 bp) on the gel electrophoresis, and the second type, which has both T and C at nucleotide position 304, were showed three fragments (482 bp, 272 bp, 210 bp) of DNA on the gel electrophoresis by restriction enzyme digestion. Eventually, characterization of the 19 isolates suggested that these isolates could harbor both the *aac(6')-Ib* gene and the variant. One of the 19 strains as an exception showed one fragment of DNA on gel electrophoresis when treated by restriction enzyme *Bts*CI (Fig. 1B). Although the *Bts*CI restriction recognition site is present in the wild-type gene sequence, it was not cut by restriction enzyme *Bts*CI. The reason for this difference is not yet understood, and requires further study.

Meanwhile, it appears that these strains, which have both T and C at nucleotide position 304, are harboring both *aac(6')-Ib* and the *aac(6')-Ib-cr* variant simultaneously. This phenomenon had not previously been seen in other studies conducted in Korea. There have been studies that noted the possibility that some plasmids carry both *aac(6')-Ib-cr* and *qnrA* (14). Therefore, we think the phenomenon we are observing may be a transitional period. In addition, the presence of *aac(6')-Ib-cr* alone substantially increased the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin (15). Also, we could confirm 47 of the total 85 (55.3%) isolates harbored the *aac(6')-Ib-cr* variant using the two methods in the study. These results could be compared with the studies of other countries. Among clinical *Escherichia coli* isolates collected in Shanghai, China, in 2000 to 2001, 51% had the cr variant of *aac(6')-Ib* (15).

In order to further prove the phenomenon of simultaneous harboring of *aac(6')-Ib* and the *aac(6')-Ib-cr* variant, we conducted further experiments through DHPLC. The DHPLC profiling patterns tended to be plural and became more complicated when performing experiments in the multiple mutant strains (16, 17). We could confirm the DHPLC conditions to be optimal when wild-type homoduplexes manifested as a single peak, and mixtures of mutant and wild-type amplicons (i.e., mixtures of heteroduplexes and homoduplexes) manifested as more peaks. All mixtures of mutant and wild-type amplicons produced patterns that were clearly distinguishable from the wild-type amplicons in this study. These profiles were repeatedly reproducible at optimal conditions. The elution times for the diagnostic peaks ranged from 5.0 to 5.5 min. There were some different profiling patterns detected between the wild-type *aac(6')-Ib* and *aac(6')-Ib-cr* variant using DHPLC. We also found that there were two profiling patterns in *aac(6')-Ib-cr* variant (Fig. 2). The strains with only T or C alone at nucleotide position 304 in the sequence analysis showed one peak on the chromatogram with DHPLC (Fig. 2a, b). On the other hand, the strains with both T and C present simultaneously at nucleotide position 304 in the sequence analysis showed more peaks on the chromatogram with DHPLC (Fig. 2c). Therefore, we also have tried to mix artificially the products, which are having only T or C alone, and then the products, which are mixed T and C by artificial means, were also showed more peaks in the chromatogram by DHPLC (Fig. 2d). They were the spitting image of the strains, which are having both T and C at nucleotide position 304. Therefore, we have confirmed that the strains that naturally have both T and C at nucleotide position 304 have been harboring both genes (*aac(6')-Ib* and the *aac(6')-Ib-cr* variant). We compared differences in the DHPLC profiling to the sequencing results; this confirmed that the DHPLC profiling corresponded with the sequencing results in the mutation pattern. From this, it is apparent that the DHPLC profiling corresponded with the genes' amino acid sequence in each case.

In order to investigate the change in the minimum inhibitory concentration (MIC) of antibiotics (specifically aminoglycoside and fluoroquinolone) brought about by *aac(6')-Ib* and *aac(6')-Ib-cr* variant when they are present in the same cell, we ana-

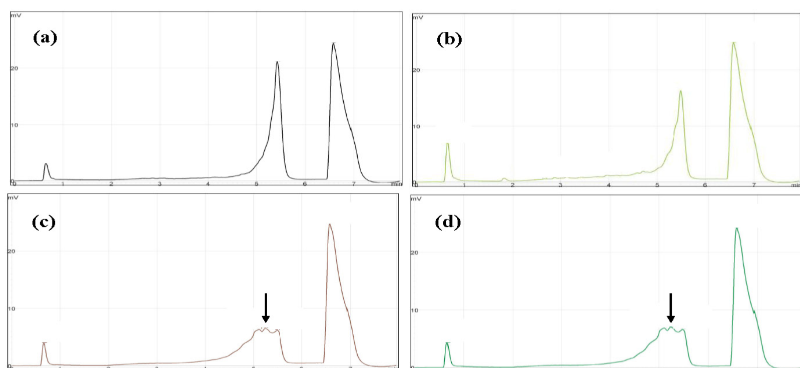

Fig. 2. Observed chromatograms patterns of *aac(6´)-Ib* and *aac(6´)-Ib-cr* gene in the DHPLC system at 61.6°C after submitting the four samples. Patterns: (a) The strains, which are having only C alone at nucleotide position 304 in the sequence analysis; (b) Wild-type, the strains, which are having only T alone at nucleotide position 304 in the sequence analysis; (c) The strains, which are having both nucleotide (T and C) at nucleotide position 304 in the sequence analysis; (d) The PCR products of *aac(6')-Ib-cr* (only C) were mixed with an equal amount of DNA amplified from an wild-type strains of *aac(6')-Ib* (only T) by artificial means. The axes (not shown) represent retention times in minutes (x) and absorbance at 260 nm (y). Two arrows indicate peaks of the mutant, respectively.

lyzed the relationship between *aac(6')-Ib-cr* variant and susceptibility to amikacin (aminoglycoside) and ciprofloxacin (fluoroquinolone) (Table 1). The susceptibility test against amikacin and ciprofloxacin was performed in all 85 strains using the broth microdilution method. The MIC mean values against amikacin and ciprofloxacin in the 38 strains with T at nucleotide position 304 (*aac(6')-Ib*) were 22.11 and 13.12 ug/ml, respectively. Among the 38 strains, 11 (28.9%) were resistant to amikacin and 35 (92.1%) were resistant to ciprofloxacin. The MIC mean values against amikacin and ciprofloxacin in the 28 strains with C at nucleotide position 304 (*aac(6')-Ib-cr* variant) were 22.6 and 14.47 ug/ml, respectively. Among the 28 strains, 8 (28.6%) were resistant to amikacin and 24 (85.7%) were resistant to ciprofloxacin. The MIC mean values against amikacin and ciprofloxacin in the 19 strains with both T and C simultaneously at nucleotide position 304 (*aac(6')-Ib-cr* variant) were 22.11 and 11.76 ug/ml, respectively. Among the 19 strains, 4 (21.1%) were resistant to amikacin and 15 (78.9%) were resistant to ciprofloxacin (Table 1). As shown in the Table 1, there is no difference between clinical isolates carrying *aac(6')-Ib* and *aac(6')-Ib-cr* genes with respect to the MIC mean values against ciprofloxacin in our results. Namely, no association with (or impact on) MIC was found between the strains, which have only T or only C at nucleotide position 304, and the strains with both T and C at nucleotide position 304. The reasons were known to have another resistance mechanisms against fluoroquinolones, such as mutations in quinolone-resistance determining regions (QRDRs) of *gyrA* or efflux pumps. Alteration of the *gyrA* subunit of DNA gyrase has an important role in conferring high-level quinolone resistance in gram-negative bacteria such as *E. coli* and *Neisseria gonorrhoeae* (18). In *E. coli*, several mutations have been identified in the *gyrA* gene. Among these, mutations at Ser-83 and Asp-87 have been found with a higher frequency in quinolone resistant *E. coli* clinical isolates than in susceptible isolates (19). The genes for multidrug efflux pump *oqxAB*, which is active on fluoroquinolones, were detected in human clinical isolates on a plasmid in *E. coli* and on the chromosome of *K. pneumoniae* (20). Therefore, in order to find the other resistance mechanisms against fluoroquinolones, we performed tests to characterize genetic mutation in QRDR of *gyrA* gene and *oqxAB* gene against used isolates in this study.

In our results, substitutions at codons 83 (Ser→Leu or Ile, Phe) and/or 87 (Asp→Asn or Ala) in the *gyrA* gene were detected in 52.6% (20/38) of the strains (*E. coli* 14 and *K. pneumoniae* 6) carrying *aac(6')-Ib* gene (data not shown). Furthermore, the strains that *oqxA* and/or *oqxB* gene harbor were detected in 15.8% (10/38) of the strains (*K. pneumoniae*) carrying *aac(6')-Ib* gene (data not shown). Due to these reasons (mutations in QRDR of *gyrA* or having efflux pump *oqxAB*), we think that the strains carrying *aac(6')-Ib* gene only were resistant to ciprofloxacin in this study.

There are two main methods that can detect *aac(6')-Ib-cr* variant in clinical strains, sequence analysis and the restriction enzyme method. However, there are some strains that cannot be analyzed precisely with only one method when detecting *aac(6')-Ib-cr* variant. Although it is not easy to perform experiments, these two methods (sequence analysis and restriction enzyme method) should be used together when possible for discrimination of *aac(6')-Ib-cr* variant. Therefore, we recommend that the strains which were suspected of *aac(6')-Ib-cr* variant positive in the clinical isolates should be used both the sequence analysis and the restriction enzyme method.

MATERIALS AND METHODS

Bacterial isolates

During the period from January 2006 to December 2006, we selected the 85 strains (*E. coli* 50, *K. pneumoniae* 35) which can produce enzyme (ESBL, Extended Spectrum Beta Lactamase) and were the high resistant to ciprofloxacin simultaneously from clinical isolates in the Kyung-Hee Medical center in the Republic of Korea. Due to the high resistant to ciprofloxacin, the strains were suspected of *aac(6')-Ib-cr* variant.

DNA isolation and PCR amplification

DNA was isolated from *E. coli* and *K. pneumoniae* using QIAGEN Plasmid Prep Kit (Qiagen, Germany). *aac(6')-Ib* was amplified by PCR with primers 5'-TTGCGATGCTCTATGAGTGGCTA and 5'-CTCGAATGCCTGGCGTGTTT to produce a 482 bp product. PCR conditions were 94°C for 45 sec, 53°C for 60sec, and 72°C for 60 sec for 40 cycles. *gyrA* was amplified by PCR with primers 5'-AAATCTGCCCGTGTCGTTGGT and 5'-GCCATACCTACGGCGATACC to produce a 344 bp product. PCR conditions were 94°C for 60 sec, 55°C for 45 sec, and 72°C for 60 sec for 30 cycles. *oqxA* was amplified by PCR with primers 5'-CTCGGCGCGATGATGCT and 5'-CCACTCTTCACGGGAGACGA to produce a 392 bp product. PCR conditions were 94°C for 45 sec, 57°C for 45 sec, and 68°C for 60 sec for 34 cycles. *oqxB* was amplified by PCR with primers 5'-TTCTCCCCCGGCGGGAAGTAC and 5'-CTCGGCCATTTTGGCGCGTA to produce a 512 bp product. PCR conditions were 94°C for 45 sec, 64°C for 45 sec, and 72°C for 60 sec for 32 cycles.

Sequence analysis, cloning and digestion by restriction enzyme

Direct sequencing of the PCR products and clones with specific primer 5'-TTGCGATGCTCTATGAGTGGCTA to identify *aac(6')-Ib-cr* variant. Sequences were read using an ABI 3130XL DNA genetic analyzer (Applied Biosystems, USA). PCR products of *aac(6')-Ib* and *aac(6')-Ib-cr* variant were gel-purified and ligated into a pGEM T-easy vector (Promega, USA) with T4 DNA ligase. Positive clones containing inserts of predicted size were sequenced using T7 and SP6 primers. All PCR products positive for *aac(6')-Ib* were further analyzed by digestion with *Bts*CI (New England Biolabs, UK).

DHPLC analysis

In vitro mutants with defined mutations in *aac(6')-Ib* and *aac(6')-Ib-cr* variant, as determined by nucleotide sequence analysis, were selected for examination using DHPLC. The PCR products of these *in vitro* samples were then run as a selected collection on a DHPLC DNA Sep column under partial heat denaturation. For DHPLC analysis, PCR products (1 μg) were mixed with an equal amount of DNA amplified from a wild-type standard, and denatured at 95°C for 5 min and then allowed to slowly reanneal for formation of heteroduplexes. It is this combination of ion exchange and partial denaturation. DHPLC was carried out using Transgenomic Wave DHPLC equipment (Transgenomic, USA) and a DNA Sep HT cartridge column (Transgenomic, USA). Analysis was carried out at a flow rate of 0.9 ml/min. Elution of DNA from the column was detected by absorbance at 260 nm. DHPLC data analysis was based on subjective comparison of sample and reference chromatograms. Any sequence variation in PCR products results in reduced column retention time and changes in peak profiles.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of the test organisms were determined against ceftazidime, cefepime, cefotaxime, aztreonam, amikacin and ciprofloxacin using the broth microdilution method (21). The MIC breakpoints were established by the CLSI guideline in this study.

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