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Original Article

Dissemination of Plasmid-mediated *qnr*, *aac(6')-Ib-cr*, and *qepA* Genes Among 16S rRNA Methylase Producing *Enterobacteriaceae* in Korea

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Plasmid-mediated quinolone resistance (PMQR) genes: *qnr*, *aac(6')-Ib-cr*, and *qepA* were investigated among 153 *armA* and 51 *rmtB*-positive transconjugants and their 204 clinical isolates of *Enterobacteriaceae*. Overall, *qnrB4* and *aac(6')-Ib-cr* genes were identified in 52.3% (63 *K. pneumoniae*, 10 *E. coli*, 4 *E. cloacae*, and 3 *E. aerogenes*) and 24.8% (16 *K. pneumoniae*, 8 *E. coli*, 6 *S. marcescens*, 4 *E. cloacae*, 3 *C. freundii* and 1 *K. oxytoca*) of 153 *armA*-positive isolates, respectively. Four isolates of *K. pneumoniae* and two isolates of *E. coli* positive for *armA* co-harbored both *qnrB4* and *aac(6')-Ib-cr*. The *qepA* gene was detected in 11.8% (5 *E. coli* and 1 *K. pneumoniae*) of 51 *rmtB*-positive clinical isolates and their transconjugants. Southern hybridization confirmed the co-localization of *qepA* and *rmtB* on a large conjugative plasmid of size between 90 to 170 kb. Inc replicon typing showed that *qnrB4/6*, *aac(6')-Ib-cr*, and *qepA* genes were principally disseminated by IncFIIAs, IncL/M, and IncF plasmids, respectively. This study constitutes the first report of the three known PMQR genes among the 16S rRNA methylase producing *Enterobacteriaceae* isolates of human origin from Korea.

Key Words: *qnr*; *aac(6')-Ib-cr*; *qepA*; *armA*; *rmtB*; Resistance

INTRODUCTION

Quinolone resistance in *Enterobacteriaceae* mostly results from chromosomal mutations in genes coding for DNA gyrase and topoisomerase IV and changes in outer membrane and efflux proteins or in their regulatory mechanisms (1). However, since the first plasmid-mediated quinolone resistance (PMQR) gene was reported in 1998 for a *Klebsiella pneumoniae* isolate from the United States (2), three PMQR genes have been discovered: *qnr*, *aac(6')-Ib-cr*, and *qepA*. The *qnr* genes encode proteins of the pentapeptide repeat family that directly protects DNA gyrase and topoisomerase IV from inhibition by quinolone (3, 4). At least three families of *qnr* genes, *qnrA*, *qnrB*, and *qnrS*, have been reported worldwide in various enterobacterial species (2, 5~7) and 6 variants of *qnrA*, 19 variants of *qnrB*, and 3 variants of *qnrS* genes are known (8). The *aac(6')-Ib-cr* gene encodes a new variant of common aminoglycoside acetyltransferase that acetylates piperazinyl substituent of some fluoroquinolones, including norfloxacin and ciprofloxacin (9), and thereby reduces their antibacterial activities. It has been reported to be geographically widespread (10, 11). It was first reported in 2003 and confers 2- to 4-folds increase in MICs (12). The novel *qepA* gene encodes an efflux pump belonging to the major facilitator superfamily. It confers a 32-to 64-fold increase of hydrophilic fluoroquinolone MIC values (13). It was identified on plasmid pHPA of *Escherichia coli* C316, which was isolated in 2002 from the urine of an inpatient in Japan (13),

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and on plasmid pIP1206 from *E. coli* 1450, which was isolated in a Belgian hospital (14). It resides on a putative transposable element along with *rmtB* gene flanked by two copies of IS*26* element on the same transferable plasmid. Recently, *qepA2* gene located on a 90 kb mobilizable plasmid but associated neither to *rmtB* gene nor to IS*26* element, in contrast to those reported from Japan and Belgium, has been reported in a CTX-M-15-positive *E. coli* from France (15).

A series of special methylases that protect microbial 16S rRNA has been identified in several nosocomial pathogens and these enzymes are capable of conferring extraordinary high levels of resistance (MIC >512 mg/l) against most clinically important aminoglycosides including amikacin, isepamycin, arbekacin, kanamycin, tobramycin, and gentamicin (16~18). Since the first identification of gene encoding 16S rRNA methylase, *rmtA*, from the *Pseudomonas aeruginosa* isolate in 2003, four major 16S rRNA methylases, *armA*, *rmtB*, *rmtC*, and *rmtD* have been reported (16~19). In a previous study, we reported a wide spread of *armA* among various species of *Enterobacteriaceae* and spread of *rmtB* among *K. pneumoniae* and *E. coli* at a University Hospital, in South Korea. In addition, it was found that transferable plasmids which confer high level resistance to amikacin and carry 16S rRNA methylase genes-*armA* and *rmtB* were associated to quinolone resistance (20). Moreover, to date very few reports are available on the occurrence of novel *qepA* gene and no data about the prevalence of all the three PMQR genes among the clinical isolates of bacteria producing 16S rRNA methylase from human around the world have been published (3, 15). Therefore, in this study, the distribution of *qnr*, *aac(6')-Ib-cr*, and *qepA* genes among ArmA- or RmtB-producing *Enterobacteriaceae* isolates of human were investigated. In addition, to know the mechanism of dissemination of the genes, conjugative plasmids carrying PMQR and 16S rRNA methylase genes were identified by a PCR-based replicon typing of the major plasmid incompatibility groups among *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial strains

One-hundred and fifty three *armA*- and 51 *rmtB*-positive transconjugants and their 204 respective clinical isolates of *Enterobacteriaceae* collected consequently between 2001 and 2006 at Kyungpook National University Hospital in the Republic of Korea, were investigated. Species identity, transferability of amikacin resistant determinants, and distribution of 16S rRNA methylases (*rmtA*, *rmtB*, *rmtC*, and *armA*), were previously characterized (20).

PCR amplification and sequencing

DNA templates were prepared by boiling method. Detection of *qnrA*, *qnrB*, and *qnrS* genes was done by multiplex PCR using primers and conditions as described previously by Robicsek et al. (21). Subtyping of *qnrB* gene was done by PCR restriction fragment length polymorphism (RFLP) as described previously (22). A 415 bp fragment of *aac(6')-Ib* was amplified as described previously (20). The primer set used was those described by Shi et al. (23) for *aac(6')-Ib*. All positive amplicons were subsequently digested with *Fok*I (New England Biolabs, Ipswich, MA, USA) to identify *aac(6')-Ib-cr*, which lacks the *Fok*I restriction site present in the wild-type gene. Digestion of 415 bp fragment of *aac(6')-Ib* gene, in contrast, produced two smaller fragments of size 217 bp and 198 bp. For amplification of *qepA* gene, primers T-qepA-F (5'-GGACATCTACGGCT-TCTTCG-3') and T-qepA-R (5'-AGCTGCAGGTACTGC-GTCAT-3') were used to produce a 720 bp amplicon. The PCR conditions used for *qepA* were as follows: initial denaturation at 94℃ for 5 min, followed by 30 cycles of amplification at 94℃ for 1 min, annealing at 59℃ for 30 sec, and extension at 72℃ for 1 min, with final extension at 72℃ for 5 min. The PCR product thus obtained from one *qepA* positive *E. coli* strain was sequenced using automated ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA) using the same primers used for PCR amplification. Sequence analysis and comparison with known sequences were performed with the BLAST programs

at the National Center for Biotechnology Information (http//www.ncbi.nlm.nih.gov/BLAST).

Inc replicon typing

A PCR-based Inc replicon typing was done using plasmid DNA templates as described previously by Carattoli et al. (24). The major Inc replicons were amplified by 5 multiplex- and 3 simplex-PCRs using eighteen pairs of primers, recognizing FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons. As positive controls of PCR reactions, R27 (HI1), R478 (HI2), R483 (I1), R446b (L/M), RN3 (N), Tp181 (FIme), RS-a (W), RP4 (P), R40a (A/C), Rts1 (T), R124 (FIV), R387 (K), R16 (B/O), and R6K (X) were used.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of the clinical isolates and their transconjugants were determined by agar dilution method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) for agar dilution, using Mueller-Hinton agar (25). The antimicrobial agents included were gentamicin and kanamycin (DUCHEFA, Haarlem, Netherland), amikacin and trimethoprim (ICN Biomedicals, Irvine, CA, USA), streptomycin (Sigma Chemical Co., St. Louis, MO, USA), ampicillin (USB, Cleveland, OH, USA), cefoxitin (Sigma), cefotaxime (Sigma), cefepime (Boryung Inc., Seoul, Korea), aztreonam (Sigma), ceftazidime (Sigma), ciprofloxacin (Fluka, Buchs, Switzerland), chloramphenicol (Sigma), tetracycline (Sigma), and sulfamethoxazole (Sigma). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

Plasmid preparation and Southern hybridization

Plasmid DNAs were isolated from the *aac(6')-Ib-cr* and *armA*-positive as well as *qepA*- and *rmtB*-positive transconjugants by the alkaline lysis method (26). The plasmids from the *qepA*- and *rmtB*-positive transconjugants were digested with *Eco*RI (Takara, Shiga, Japan) and *Apa*I (Takara) respectively. The whole plasmids as well as digested plasmid DNAs were electrophoresed separately through 0.7% agarose gel, and were transferred onto a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) by capillary transfer. They were then subjected to Southern hybridization using probes specific for *qepA* and *rmtB* genes. The whole plasmids from the *aac(6')-Ib-cr* and *armA*-positive transconjugants were also subjected to hybridization with *aac(6')-Ib* and *armA* probes specific for *aac(6')-Ib-cr* and *armA* genes, respectively. A digoxigenin DNA labeling and detection kit (Boehringer

Table 1. Distribution of plasmid-mediated *qnr*, *aac(6')-Ib-cr*, and *qepA* genes among the clinical isolates of *Enterobacteriaceae* carrying *armA* or *rmtB* genes

Species and PMQR genes they carried	No. of strains carrying indicated genes		Total
	armA (n=153)	*rmtB* (n=51)	
K. pneumoniae (n=90)			
qnrB4	63		63
qnrB6	1		1
aac(6')-Ib-cr	16	5	21
qnrB4 and *aac(6')-Ib-cr*	4		4
qepA		1	1
K. oxytoca (n=1)			
aac(6')-Ib-cr	1		1
E. coli (n=25)			
qnrB4	10		10
aac(6')-Ib-cr	8		8
qepA		4	4
qnrB4 and *aac(6')-Ib-cr*	2		2
qepA and *aac(6')-Ib-cr*		1	1
Enterobacter cloacae (n=8)			
qnrB4	4		4
aac(6')-Ib-cr	4		4
Enterobacter aerogenes (n=3)			
qnrB4	3		3
Serratia marcescens (n=6)			
aac(6')-Ib-cr	6		6
Citrobacter freundii (n=3)			
aac(6')-Ib-cr	3		3
Total	125	11	136

Mannheim) was used according to the manufacturer's instructions.

Pulsed-field gel electrophoresis

PFGE analysis of *Xba*I (Roche Diagnostics GmbH, Roche Applied Science, Nonnenwald, Penzberg, Germany) digested genomic DNA of clinical isolates of *Enterobacteriaceae* carrying *aac(6')-Ib-cr* or *qepA* genes was performed with a CHEF DRIII system (Bio-Rad Laboratories, Hercules, CA, USA), in a 0.5 × TBE buffer, according to the instruction manual. Electrophoresis was performed at 6 V/cm for 20 hr at 12℃ with pulse times ramped from 5 sec to 40 sec. A phage lambda DNA ladder (Bio-Rad) was used as a size marker. The banding patterns were then analyzed using Gel Compar II software program (Applied Maths, Sint-Martens-Latem, Belgium).

RESULTS

Distribution of *qnr*, *aac(6')-Ib-cr*, and *qepA* genes

The PMQR genes were detected in 81.7% and 73.2% of 153 *armA*-positive clinical isolates of *Enterobacteriaceae* and their transconjugants, respectively. Similarly, they were found in 21.6% and 11.8% of 51 *rmtB*-positive clinical isolates and their transconjugants, respectively. Among the 153 *armA*-positive isolates investigated, *qnr* genes were identified alone or in combination with other PMQR genes in 87 (56.9%) of them (Table 1) and their 77 transconjugants (Table 2). The *qnr* genes detected include 86 *qnrB4* and one *qnrB6* which were most often found among *K. pneumoniae* (68/90, 75.6%) followed by *E. coli* (12/25, 48.0%). No *qnr* gene was detected among the *rmtB*-positive isolates. The *aac(6')-Ib-cr* gene was detected alone or in combination in 44 (28.8%) of 153 *armA*-positive isolates and their 35 transconjugants. It was also detected in six *rmtB*-positive isolates but none of their transconjugants was positive for it. Among the *armA*-positive isolates, four isolates of *K. pneumoniae* and two isolates of *E. coli* were positive for both *qnrB4* and *aac(6')-Ib-cr* but only *qnrB4* gene transferred simultaneously with *armA* to the recipient *E. coli* strain. The *qepA* gene was detected in 11.8% of *rmtB*-positive clinical isolates and their respective transconjugants. It was found alone in four *E. coli* and one *K. pneumoniae* isolates, whereas *qepA* and *aac(6')-Ib-cr* co-existed in a *rmtB*-positive *E. coli* strain. However, only *qepA* transferred simultaneously with *rmtB* to the recipient *E. coli* strain. The nucleotide sequence of 720 bp *qepA* PCR product amplified from *E. coli* strain 05K1330 was identical to that of the first reported *qepA* gene (GenBank accession no. **AB263754**) identified in plasmid pHPA derived from *E. coli* C316 from Japan.

Inc/ replicon typing

In order to identify the conjugative plasmids carrying simultaneously PMQR and *armA* or *rmtB* genes, PCR-based replicon typing was done. Of the 77 transconjugants that carry *armA* and *qnr* genes simultaneously, IncFIIAs was detected in 16 transconjugants but none of the Inc

Table 2. Inc/replicon types of conjugative plasmids carrying plasmid-mediated quinolone resistance genes and *armA* or *rmtB* genes

Plasmid-mediated quinolone resistance genes	No. of transconjugants carrying plasmids having indicated genes and Inc plasmid						Total
	armA (n=153)					*rmtB* (n=51)	
	A/C (n=7)	A/C, I1-Iγ (n=2)	FIIAs (n=20)	L/M (n=37)	NI[a] (n=75)	F (n=6)	
qnrB4			15		61		76
qnrB6			1				1
Aac(6')-Ib-cr	2	1		32			35
qepA						6	6
Total	2	1	16	32	61	6	118

[a] NI, not identified

replicon types studied was detected in the remaining 61 transconjugants (Table 2). IncL/M plasmid was detected in 32 transconjugants positive for both *aac(6')-Ib-cr* and *armA* genes. IncF was detected in all the six transconjugants that simultaneously carried *qepA* and *rmtB* genes. Thus, among the *armA*-positive transconjugants *qnr* genes were disseminated by IncFIIAs and Inc (unidentified) plasmids and *aac(6')-Ib-cr* genes mainly by IncL/M plasmids, while among the *rmtB*-positive transconjugants *qepA* genes were found to be disseminated by IncF plasmid only.

Antimicrobial susceptibility testing

The MIC_{50} of nalidixic acid and ciprofloxacin for 77 *qnrB*-positive transconjugants were 16 (MIC range, 8 to 32) and 0.25 (MIC range, 0.03 to 0.25) μg/ml, respectively. Similarly, the MIC_{50} of nalidixic acid and ciprofloxacin for 35 *aac(6')-Ib-cr*-positive transconjugants were 8 and 0.06 μg/ml, respectively representing 4-fold higher ciprofloxacin MICs as compared to that of recipient *E. coli* RG488 strain. A majority (5/6, 83.3%) of transconjugants carrying *qepA* showed 16-fold increase in the MICs for ciprofloxacin, relative to that of recipient *E. coli* RG488 strain. On the other hand, except in one, there was no difference in increase in MICs among the transconjugants carrying *aac(6')-Ib-cr* or *qepA* for nalidixic acid, relative to that of the recipient strain. The MICs for amikacin, kanamycin, gentamicin and tobramycin were >256 μg/ml for all the transconjugants carrying *armA* or *rmtB* genes

Plasmid and Southern blot analysis

Plasmid DNA was extracted from the 12 transconjugants positive for *aac(6')-Ib-cr* and *armA* genes. Nine transconjugants carried a single large plasmid of size around 90 kb, two of them carried one more plasmid of much smaller size plasmid in addition to 90 kb plasmid, and one of them carried a single large plasmid of size around 170 kb. Southern hybridization analysis demonstrated that the *aac(6')-Ib-cr* and *armA* genes were located on a single large plasmid for each transconjugant. Isolation of plasmid DNA from four transconjugants positive for *qepA* and *rmtB* also revealed a single large plasmid of size between 90 and 170 kb, except in one (AKp5681JR) which also harbored two more plasmids of smaller size (Fig. 1A). Southern hybridization of whole plasmids using probes specific for *qepA*

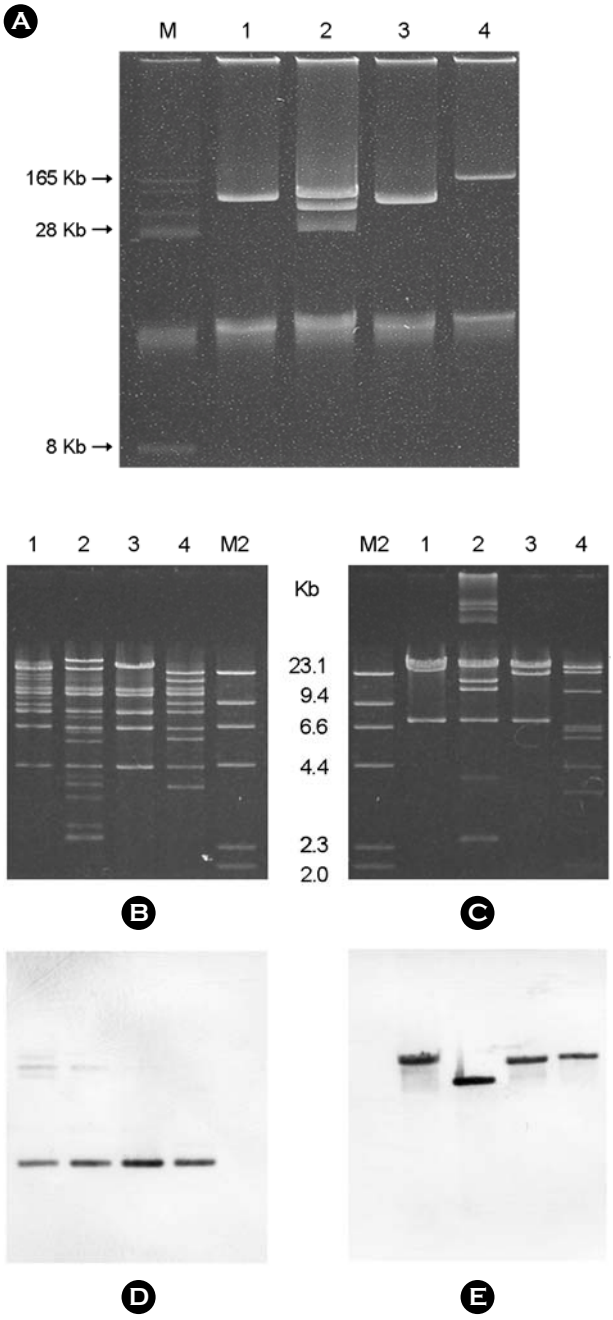

Figure 1. Gel-electrophoresis of plasmid DNAs extracted from transconjugants positive for *qepA* and *rmtB* **(A)** RFLP of Plasmid DNAs extracted from transconjugants carrying *qepA* and *rmtB* after digestion with *Eco*RI **(B)** and *Apa*I **(C)**. Southern hybridizations with *qepA* probe **(D)** and with *rmtB* probe **(E)**. Lanes: M, BAC-tracker™ supercoiled marker; M2, λ HindIII marker 2; 1, AKp5501JR; 2, AKp5681JR; 3, AKp5781JR; and 4, AKp5931JR.

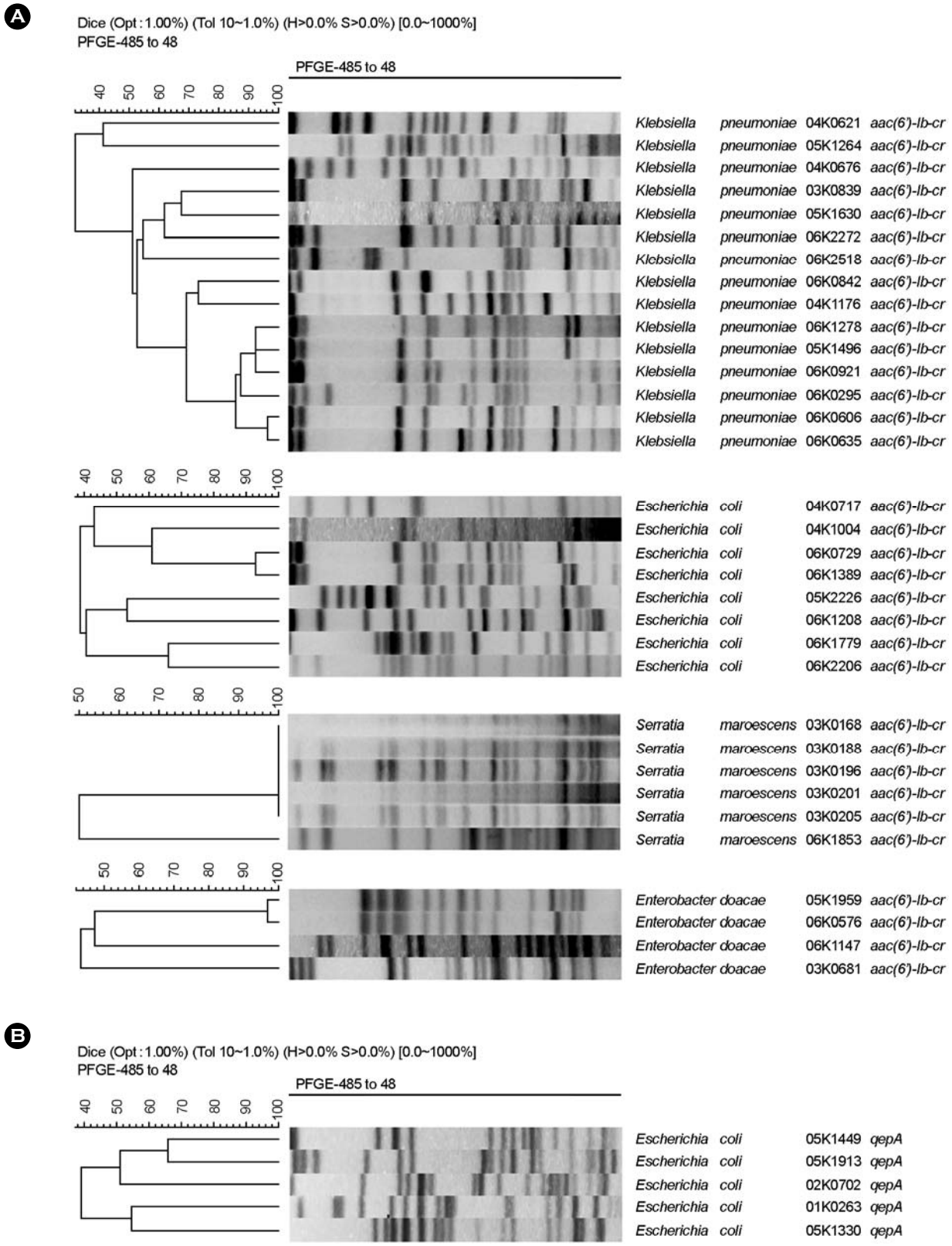

Figure 2. Dendrogram generated by Gel Compar II showing the genomic relatedness of clinical isolates *Enterobacteriaceae* carrying *armA* and *aac(6')-Ib-cr* **(A)** and isolates carrying *qepA* and *rmtB* **(B)** determined by PFGE.

and *rmtB* genes revealed that these determinants were apparently co-localized and co-carried on the same plasmid. The RFLP patterns produced by digestion of plasmids from the transconjugants with *Eco*RI or *Apa*I were not similar (Fig. 1B & C). Nevertheless, hybridization of plasmid DNA fragments with *qepA* probe after digestion with *Eco*RI revealed that *qepA* gene was located on a same ca. 4.5 kb DNA fragment irrespective of plasmid size or species from which the transconjugants were derived (Fig. 1D). Similarly, hybridization with *rmtB* probe after digestion with *Apa*I identified localization of *rmtB* gene on a same ca. 23 kb plasmid fragment, except in one of the strains (Fig. 1E).

PFGE analysis

*Xba*I-PFGE was used to investigate the genetic relatedness of 33 (15 *K. pneumoniae*, 8 *E. coli*, 6 *S. marcescens*, and 4 *E. cloacae*) *aac(6')-Ib-cr*- and *armA*-positive *Enterobacteriaceae* isolates that solely carried transferable *aac(6')-Ib-cr* genes and five *qepA*-positive *E. coli* isolates. The PFGE patterns derived from the *K. pneumoniae*, *E. coli* and *E. cloacae* were heterogeneous among the same species and revealed a great genomic diversity among them. However, the PFGE patterns derived from 5 out of six *S. marcescens* were indistinguishable, indicating they belonged to the same clone (Fig. 2A). On the other hand, five different major patterns were identified among 5 *E. coli* isolates, indicating that the spread of *qepA* was not due to the spread of a specific *E. coli* clone (Fig. 2B).

DISCUSSION

To date, very few epidemiological surveys have reported the prevalence of all the three PMQR genes, simultaneously from the same set of clinical isolates of *Enterobacteriaceae* (27). Even though *qnr* genes have been reported repeatedly worldwide among various enterobacterial species, the *aac(6')-Ib-cr* genes have been reported less often than *qnr* genes although thought to be geographically widespread (7, 10, 11), and *qepA* genes have been reported so far only from Japan, Belgium, France, and China and are mostly limited to *E. coli* strains (13~15, 28~30). Recently, it has been reported in two *E. coli* strains and more recently in two clonally unrelated *E. aerogenes* from South Korea (30, 31). The present study demonstrated a high prevalence of three PMQR genes: *qnr*, *aac(6')-Ib-cr*, and *qepA* among 16S rRNA methylase producing *Enterobacteriaceae* in Korea. Overall, *qnrB4* and *aac(6')-Ib-cr* genes were identified in 80 (63 *K. pneumoniae*, 10 *E. coli*, 4 *E. cloacae*, and 3 *E. aerogenes*) and 38 (16 *K. pneumoniae*, 1 *K. oxytoca*, 8 *E. coli*, 6 *S. marcescens*, 4 *E. cloacae*, and 3 *Citrobacter freundii*) of 153 *armA*-positive clinical isolates, respectively. Four isolates of *K. pneumoniae* and 2 isolates of *E. coli* positive for *armA* co-harbored both *qnrB4* and *aac(6')-Ib-cr* but only *qnrB4* transferred simultaneously with *armA* to the recipient *E. coli* strain, indicating that these two PMQR genes were not located on the same plasmid. The *qepA* gene was identified in six (5 *E. coli* and 1 *K. pneumoniae*) *rmtB*-positive clinical isolates, of which one *E. coli* co-carried *aac(6')-Ib-cr* gene in addition to it. Thus, *qnr* was more common than *aac(6')-Ib-cr* or *qepA* genes among the 16S rRNA methylase producing *Enterobacteriaceae* in Korea. In our previous study (22), it was found that 72.5% of 135 *qnrB*-positive *Enterobacteriaceae* isolates simultaneously carried *armA*, while in the present study the prevalence of *qnrB* among the *armA*-positive *Enterobacteriaceae* isolates was found to be 52.9%. Although these two studies were focused on two different things, *qnrB* gene was found associated with *armA* but not with *rmtB* or other 16S rRNA methylase genes. Recently, Ma et al. (27) reported a high prevalence of PMQR genes: *qnr* (7.9%), *aac(6')Ib-cr* (18.8%), and *qepA* (15.8%) among 101 ceftiofur-resistant *Enterobacteriaceae* strains isolated from animals in China, like in our study but unlike our findings they found that *aac(6')-Ib-cr* and *qepA* more common than *qnr* genes. Since *aac(6')-Ib-cr* was first described in 2003 (7) on the plasmid harboring the CTX-M-15 extended-spectrum β-lactamase, it has been detected in isolates of *Enterobacteriaceae* from various countries (10). Park et al. (11) reported *aac(6')-Ib-cr* in 15 (32%) of 47 *E. coli* isolates, 17 (16%) of 106 *K. pneumoniae*, isolates and 12 (7.5%) of 160 *Enterobacter* isolates with a ciprofloxacin MIC of $\geq$ 0.25 μg/ml and reduced susceptibility to ceftazidime from

the United States, which is more or less similar to our findings. Similarly, Avgustin et al. (10) demonstrated presence of *aac(6')-Ib-cr* on plasmids from 25 transconjugants for which the ciprofloxacin MIC was higher than 0.008 μg/ml derived from a collection of 74 ESBL-producing *K. pneumoniae* isolates from Slovenia. In contrast to our findings, Liu et al. (29) reported a high prevalence of *qepA* (58.8%) and *aac(6')Ib-cr* (12.5%) genes among the 48 RmtB-producing *E. coli* isolates from pigs in China.

In this study, all the plasmids derived from transconjugants carrying *qnrB* or *aac(6')-Ib-cr* genes were always *armA* positive, while those plasmids carrying *qepA* were always *rmtB* positive. Our previous study found that *armA* and *qnrB4* genes were co-localized, co-carried, and co-transferred along with other ESBL and/or AmpC-type β-lactamase genes on the same large conjugative plasmid (22). The association between *qepA* and *rmtB* genes in this study is explained by physical linkage of *qepA* with *rmtB* genes that was reported in the previous studies (13, 14). Although more than one type of PMQR gene co-existed in 7 out of 204 clinical isolates, none of the transconjugants carried plasmids with more than one type of PMQR gene which indicates that only a particular PMQR gene is located on and disseminated by a particular kind of conjugative plasmid. Furthermore, the *qnrB* (*qnrB4* and *qnrB6*) genes were detected only in *armA*-positive plasmids of incompatibility groups IncFIIAs and Inc (unidentified), the *aac(6')-Ib-cr* genes were identified in *armA*-positive IncL/M plasmids except in the three transconjugants, and *qepA* gene was found only in *rmtB*-positive IncF plasmid. Thus, it is proposed that among the 16S rRNA methylase producing isolates of *Enterobacteriaceae*, a specific PMQR gene is disseminated through spread of conjugative plasmid of specific Inc type or plasmids of limited Inc types. In our previous study (22), plasmids on which *qnrB4/B6* genes circulated were found to belong mainly to IncFIIAs group. However, a little is known so far about plasmids on which *aac(6')-Ib-cr* gene circulates although the plasmid on which *qepA* circulates has been reported in number of previous studies. The plasmid pHPA isolated from *E. coli* C316 from Japan which harbored *qepA1* belonged to IncFII group and the plasmid pIP1206 (ca. 100 kb) detected in *E. coli* strain 1540 from a Belgian Hospital belonged to IncFI group (14, 21). Similarly, Cattoir et al. (15) reported that the plasmid pQep (ca. 90 kb) isolated from a CTX-M-15-positive *E. coli* from France which harbored *qepA2* belonged to IncFI group.

The PMQR gene-positive plasmids in the transconjugants conferred varying degree of fluoro/quinolone resistance depending upon the type of PMQR genes they harbored. The findings of this study revealed that the highest fold increase in MICs for ciprofloxacin among the transconjugants was due to presence of *qnr*, next highest due to *qepA* and the least due to *aac(6')-Ib-cr* genes which confirmed the previous notion that the highest increase in MIC values of fluoroquinolone results owing to *qnr*, followed by *qepA* and *aac(6')-Ib-cr* genes.

The clinical characteristics of the isolates carrying *aac(6')-Ib-cr* or *qepA* showed that their spread was not due to outbreak. To confirm this, molecular characterization of 38 *aac(6')-Ib-cr*- and *armA*-positive as well as 6 *qepA*- and *rmtB*-positive isolates was done by PFGE. The PFGE analysis of 15 *K. pneumoniae*, 8 *E. coli* and 4 *E. cloacae* positive for *aac(6')-Ib-cr* and *armA* revealed great genomic diversity among them. However, five out of six *S. marcescens* isolates were clonally related. Likewise, 5 *E. coli* isolates of the total 6 *qepA*- and *rmtB*-positive isolates gave 5 different major patterns indicating that the spread of *qepA* was not due to the spread of a specific *E. coli* clone.

Quite recently *qnrD* was found in *Salmonella* isolates and *qnrC* in a clinical isolate of *Proteus mirabilis* (7, 32). This study was limited to investigation of families of *qnr* genes other than these newly discovered *qnr* families. Furthermore, this study was restricted to study of clinical isolates of *Enterobacteriaceae* in a single University hospital.

In conclusion, this study constitutes the first report of the three known plasmid-mediated quinolone resistance genes among the 16S rRNA methylase producing *Enterobacteriaceae* isolates of human origin from Korea and their prevalence was found to be high with *qnrB*, *aac(6')-Ib-cr*, and *qepA* in the decreasing order of their frequency. We also found that *qnrB4*, *aac(6')-Ib-cr*, and *qepA* genes are

principally disseminated among them through spread of IncFIIAs, IncL/M, and IncF conjugative plasmids, respectively.

REFERENCES

1) Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother 2003;51:1109-17.
2) Martinez-Martinez L, Pascual A, Jacoby GA. Quinolone resistance from a transferable plasmid. Lancet 1998; 351:797-9.
3) Tran JH, Jacoby GA. Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci USA 2002 99:5638-42.
4) Jacoby GA, Walsh KE, Mills DM, Walker VJ, Oh H, Robicsek A, Hooper DC. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob Agents Chemother 2006;50:1178-82.
5) Hata M, Suzuki M, Matsumoto M, Takahashi M, Sato K, Ibe S, Sakae K. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob Agents Chemother 2005;49:801-3.
6) Nordmann P, Poirel L. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. J Antimicrob Chemother 2005;56:463-9.
7) Wang M, Guo Q, Xu X, Wang X, Ye X, Wu S, Hooper DC, Wang M. New plasmid-mediated quinolone resistant gene, *qnrC*, found in a clinical isolate of *Proteus mirabilis*. Antimicrob Agents Chemother 2009;53: 1892-7.
8) Jacoby G, Cattoir V, Hooper D, Martínez-Martínez L, Nordmann P, Pascual A, Poirel L, Wang M. *qnr* Gene nomenclature. Antimicrob Agents Chemother 2008;52: 2297-9.
9) Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med 2006;12:83-8.
10) Ambrozic Avgustin J, Keber R, Zerjavic K, Orazem T, Grabnar M. Emergence of the quinolone resistance-mediating gene *aac(6')-Ib-cr* in extended-spectrum-beta-lactamase-producing *Klebsiella* isolates collected in Slovenia between 2000 and 2005. Antimicrob Agents Chemother 2007;51:4171-3.
11) Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC. Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob Agents Chemother 2006;50:3953-5.
12) Wang M, Tran JH, Jacoby GA, Zhang Y, Wang F, Hooper DC. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob Agents Chemother 2003;47:2242-8.
13) Yamane K, Wachino J, Suzuki S, Kimura K, Shibata N, Kato H, Shibayama K, Konda T, Arakawa Y. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother 2007;51:3354-60.
14) Perichon B, Courvalin P, Galimand M. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrob Agents Chemother 2007;51:2464-9.
15) Cattoir V, Poirel L, Nordmann P. Plasmid-mediated quinolone resistance pump QepA2 in an *Escherichia coli* isolate from France. Antimicrob Agents Chemother 2008;52:3801-4.
16) Doi Y, Yokoyama K, Yamane K, Wachino J, Shibata N, Yagi T, Shibayama K, Kato H, Arakawa Y. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. Antimicrob Agents Chemother 2004;48:491-6.
17) Galimand M, Courvalin P, Lambert T. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. Antimicrob Agents Chemother 2003;47:2565-71.
18) Wachino J, Yamane K, Shibayama K, Kurokawa H, Shibata N, Suzuki S, Doi Y, Kimura K, Ike Y, Arakawa Y. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrob Agents Chemother 2006;50:178-84.
19) Doi Y, de Oliveira Garcia D, Adams J, Paterson DL. Coproduction of novel 16S rRNA methylase RmtD

and metallo-beta-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. Antimicrob Agents Chemother 2007;51:852-6.

20) Kang HY, Kim KY, Kim J, Lee JC, Lee YC, Cho DT, Seol SY. Distribution of conjugative-plasmid-mediated 16S rRNA methylase genes among amikacin-resistant *Enterobacteriaceae* isolates collected in 1995 to 1998 and 2001 to 2006 at a university hospital in South Korea and identification of conjugative plasmids mediating dissemination of 16S rRNA methylase. J Clin Microbiol 2008;46:700-6.
21) Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA, Hooper DC. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. Antimicrob Agents Chemother 2006;50:2872-4.
22) Tamang MD, Seol SY, Oh JY, Kang HY, Lee JC, Lee YC, Cho DT, Kim J. Plasmid-mediated quinolone resistance determinants *qnrA*, *qnrB*, and *qnrS* among clinical isolates of *Enterobacteriaceae* in a Korean hospital. Antimicrob Agents Chemother 2008; 52:4159-62.
23) Shi WF, Jiang JP, Mi ZH. Relationship between antimicrobial resistance and aminoglycoside-modifying enzyme gene expressions in *Acinetobacter baumannii*. Chin Med J 2005;118:141-5.
24) Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 2005;63:219-28.
25) Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 16th Informational Supplement. M100-S16. Wayne PA: CLSI; 2006
26) Sambrook J, Russell DW. Preparation of plasmid DNA by alkaline lysis with SDS: minipreparation. In Molecular Cloning: A laboratory manual. 3rd ed. New York: Cold spring Harbour Laboratory Press; 2001. p. 1-32.
27) Ma J, Zeng Z, Chen Z, Xu X, Wang X, Deng Y, Lü D, Huang L, Zhang Y, Liu J, Wang M. High prevalence of plasmid-mediated quinolone resistance determinants *qnr*, *aac(6')-Ib-cr*, and *qepA* among ceftiofur-resistant *Enterobacteriaceae* isolates from companion and food-producing animals. Antimicrob Agents Chemother 2009; 53:519-24.
28) Yamane K, Wachino J, Suzuki S, Arakawa Y. Plasmid-mediated qepA gene among *Escherichia coli* clinical isolates from Japan. Antimicrob Agents Chemother 2008;52:1564-6.
29) Liu JH, Deng YT, Zeng ZL, Gao JH, Chen L, Arakawa Y, Chen ZL. Coprevalence of plasmid-mediated quinolone resistance determinants QepA, Qnr, and AAC(6')-Ib-cr among 16S rRNA methylase RmtB-producing *Escherichia coli* isolates from pigs. Antimicrob Agents Chemother 2008;52:2992-3.
30) Kim HB, Park CH, Kim CJ, Kim EC, Jacoby GA, Hooper DC. Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. Antimicrob Agents Chemother 2009;53:639-45.
31) Park YJ, Yu JK, Kim SI, Lee K, Arakawa Y. Accumulation of plasmid-mediated fluoroquinolone resistance genes, *qepA* and *qnrS1*, in *Enterobacter aerogenes* co-producing RmtB and class A β-lactamase LAP-1. Ann Clin Lab Sci 2009;39:55-9.
32) Cavaco LM., Hasman H, Xia S, Aarestrup FM. QnrD, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and Bovismorbificans of human origin. Antimicrob Agents Chemother 2009;53:603-8.