ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2009, p. 639–645 Vol. 53, No. 2
0066-4804/09/$08.00+0 doi:10.1128/AAC.01051-08

Prevalence of Plasmid-Mediated Quinolone Resistance Determinants over a 9-Year Period▽

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Received 4 August 2008/Returned for modification 13 October 2008/Accepted 30 November 2008

Recently, several plasmid-mediated quinolone resistance (PMQR) genes conferring low levels of quinolone resistance have been discovered. To evaluate the temporal change in the prevalence of PMQR genes over a decade in a tertiary hospital in the Republic of Korea, we selected every fifth isolate of *Escherichia coli* and *Klebsiella pneumoniae* and every third isolate of *Enterobacter cloacae* between 1998 and 2001 and between 2005 and 2006 from a collection of blood isolates. Six PMQR genes [*qnrA*, *qnrB*, *qnrC*, *qnrS*, *aac(6′)-Ib-cr*, and *qepA*] were screened by multiplex PCR and then confirmed by direct sequencing, and the *aac(6′)-Ib*-positive PCR products were digested with BtsCI to identify the *aac(6′)-Ib-cr* variant. Of 461 isolates, 37 (8%) had one of the six PMQR genes; 13 (5%) of 261 *E. coli* strains, 13 (10%) of 135 *K. pneumoniae* strains, and 11 (17%) of 65 *E. cloacae* strains. *qnrB* was the most common PMQR gene and was found as early as 1998, whereas *qnrS*, *aac(6′)-Ib-cr*, and *qepA* emerged after 2000. None of the isolates carried *qnrA* or *qnrC*. Ciprofloxacin resistance increased over time ($P < 0.001$), and the overall prevalence of PMQR genes tended to increase ($P = 0.20$). PMQR-positive isolates had significantly higher ciprofloxacin resistance and multidrug resistance rates ($P = 0.005$ and $P < 0.001$, respectively). The increasing frequency of ciprofloxacin resistance in *Enterobacteriaceae* was associated with an increasing prevalence of PMQR genes, and this change involved an increase in the diversity of the PMQR genes and also an increase in the prevalence of the mutations in *gyrA*, *parC*, or both in PMQR-positive strains but not PMQR-negative strains.

Fluoroquinolones are among the most commonly prescribed antimicrobials because of their broad-spectrum antimicrobial activity, and fluoroquinolone-resistant gram-negative pathogens have emerged worldwide. Quinolone resistance is traditionally mediated by the mutation of chromosomal genes encoding DNA gyrase and/or topoisomerase IV or by the mutation of genes regulating the expression of efflux pumps (5, 6).

It was thought that quinolone resistance could be acquired only by chromosomal mutations, until plasmid-mediated resistance to quinolones was described in a clinical isolate of *Klebsiella pneumoniae* in 1998 (12). Since then, four major groups of *qnr* determinants, *qnrA*, *qnrB*, *qnrC*, and *qnrS*, have been identified (7, 29), and two additional plasmid-mediated quinolone resistance (PMQR) genes have been described—*aac(6′)-Ib-cr*, which encodes a variant aminoglycoside acetyltransferase that modifies ciprofloxacin (21), and *qepA*, which encodes an efflux pump belonging to the major facilitator subfamily (19, 32). These PMQR determinants are increasingly being identified worldwide in clinical isolates of *Enterobacteriaceae* (4, 11, 17, 22, 23, 27) and in clinical and environmental *Aeromonas* species isolates (1, 26).

Since the report of the first horizontally transmissible element, *qnrA*, conferring resistance to quinolones, many epidemiological surveys have been reported. However, most focused on *Enterobacteriaceae* with specific resistance phenotypes, such as resistance due to extended-spectrum β-lactamases and/or reduced susceptibility to nalidixic acid or fluoroquinolones (2, 3, 10, 16, 17, 23, 28) even though PMQR genes do not themselves confer full resistance to fluoroquinolones (23). In this study, we determined the changes with time in the prevalence of all so-far-known PMQR genes in consecutive clinical *Enterobacteriaceae* isolates in a South Korean tertiary care hospital, where the frequency of ciprofloxacin resistance has continued to rise for a decade.

(This work was presented in part at the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy [ICAAC] and the 46th Annual Meeting of the Infectious Diseases Society of America [IDSA], Washington DC, 2008.)

MATERIALS AND METHODS

Bacterial isolates. Test isolates were taken from the blood isolates collection of Seoul National University Hospital, a tertiary 1,600-bed hospital in the Republic of Korea. We selected three 2-year periods (1998 to 1999, 2000 to 2001, and 2005 to 2006) in the interval from 1998 to 2006, based on resistance rates to ciprofloxacin, which represents the period before, during, and after the increase in ciprofloxacin resistance rate, respectively (Fig. 1). Every fifth consecutive isolate of *Escherichia coli* and *K. pneumoniae*, as well as every third consecutive isolate of *Enterobacter cloacae*, was included in the study.

Antimicrobial susceptibility tests. An antimicrobial disk diffusion test was carried out, in accordance with the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (14). The following 12 antibiotics were tested: amikacin, ampicillin (or cefotetan), aztreonam, cefotaxime, ceftazidime, cefuroxime, cephalothin, ciprofloxacin, gentamicin, imipenem, tobramycin, and

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▽ Published ahead of print on 8 December 2008.

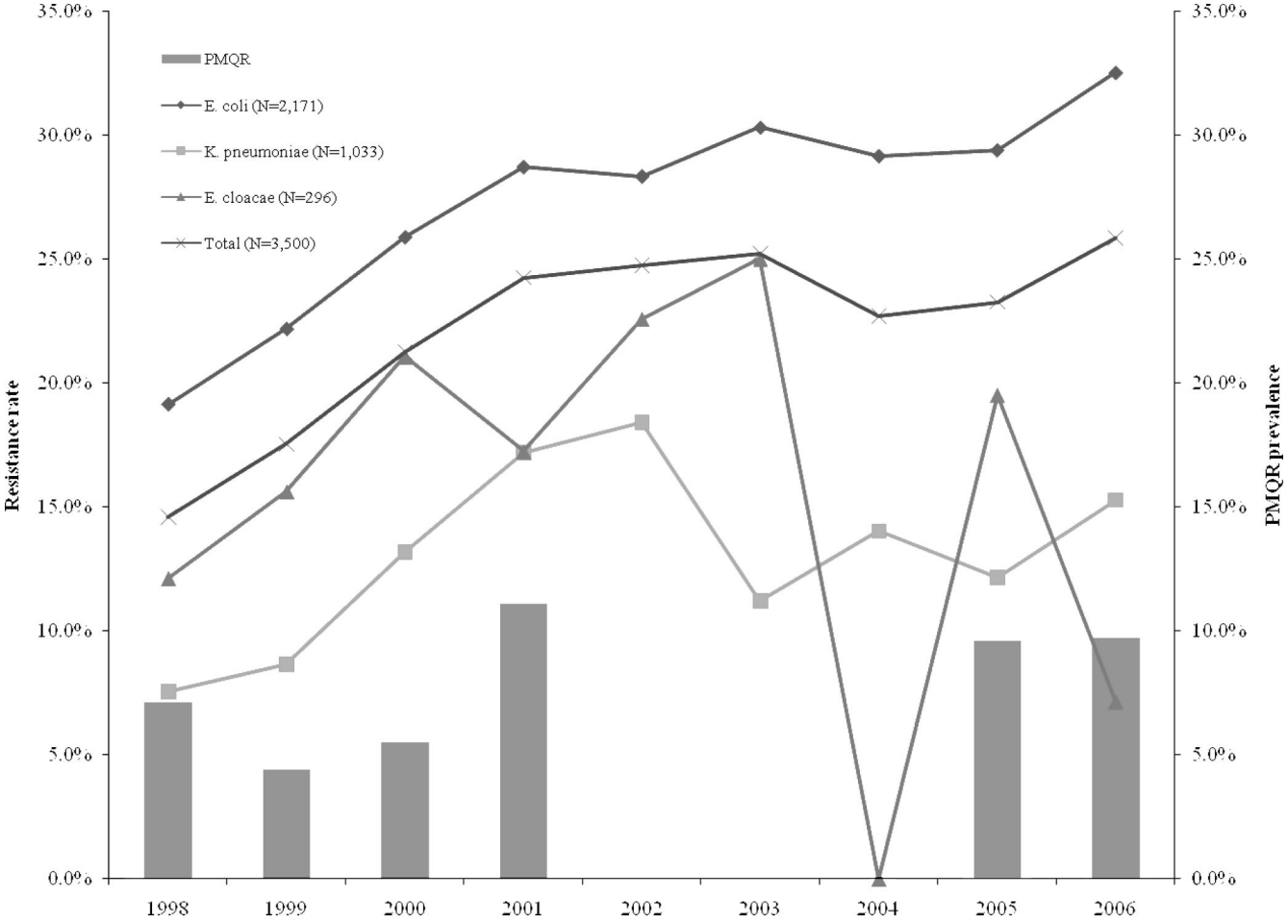

FIG. 1. Trends of ciprofloxacin resistance rates among total *E. coli*, *K. pneumoniae*, and *E. cloacae* isolates recovered from blood cultures (from 1998 through 2006), and the prevalence of the PMQR genes among 461 randomly selected isolates from 1998 to 2001 and from 2005 to 2006.

trimethoprim-sulfamethoxazole (or piperacillin-tazobactam). The breakpoints for resistance were those recommended by the CLSI (14). In addition, the MIC for ciprofloxacin was determined by Etest (AB Biodisk, Solna, Sweden). Resistance rates were calculated as the number of intermediate and resistant strains over the total number of strains. Multidrug resistance (MDR) was defined as resistance to at least three different classes of antimicrobials.

Multiplex PCR. Colonies were suspended in 50 μl of water in a microcentrifuge tube and boiled to prepare DNA templates for PCR. Pairs of primers to amplify internal fragments were designed from the sequences from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) and from the sequence of *qnrC* provided by Minggui Wang (Table 1) (29). Screening of the six PMQR determinants was carried out by two sets of multiplex PCR amplification, one for *qnrA*, *qnrB*, *qnrC*, and *qnrS* and the other for *aac(6′)-Ib* and *qepA*. In each multiplex PCR, all of the primers were added to the template DNA and PCR SuperMix high-fidelity polymerase (Invitrogen, Carlsbad, CA). Clinical isolates that had previously been confirmed to carry the *qnr* genes, *aac(6′)-Ib*, and *aac(6′)-Ib-cr* by DNA sequencing and an *E. coli* TOP10 derivative harboring *qepA* (19) were used as positive controls. Positive and negative controls were included in each PCR. Amplification products were identified by their sizes after electrophoresis on 1.8% agarose gels at 130 V for 30 min and staining with ethidium bromide. Positive results for *qnr* genes were confirmed by direct sequencing of PCR products. The *qnrB* allele number was designated based on the recent proposal for *qnr* gene nomenclature (7).

All PCR products positive for *aac(6′)-Ib* were further analyzed by digestion with BtsCI (New England Biolabs, Ipswich, MA) to identify *aac(6′)-Ib-cr*, which lacks the BtsCI restriction site present in the wild-type gene (17). The wild-type *aac(6′)-Ib* PCR product yielded 210-bp and 272-bp fragments after restriction.

Sequencing of *gyrA* and *parC*. PCR amplifications of the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* were carried out using the primers as shown in Table 1. Purified PCR products were sequenced on both strands, and then QRDR DNA sequences were compared with the sequences of *E. coli*, *K. pneumoniae*, and *E. cloacae* (GenBank accession numbers were AF052254, AF052258, and AF052256 for *gyrA* and NC000913, AF303641, and D88981 for *parC*, respectively).

Statistical analysis. The dosages of fluoroquinolone antimicrobials used in the source hospital were derived from pharmacy records over the study period. These data were converted into a number of defined daily doses (DDDs) and expressed as antimicrobial-use densities (the number of DDDs per 1,000 patient days), following the recommendation of the World Health Organization (WHO) (http://www.whocc.no/atcddd/).

Differences in proportions were compared using the χ^2 test or Fisher's exact test, as appropriate, and temporal trends were examined with the Mantel-Haenszel χ^2 test. The relation between ciprofloxacin resistance and prevalence of PMQR genes was assessed by calculating Spearman's correlation coefficient and the corresponding *P* value. All tests of significance were two-tailed, with the α value set at 0.05. All statistical analyses were done using SPSS software (SPSS, Chicago, IL).

RESULTS

Overall, 461 isolates were included in this study. Among them, 65 were provisionally identified as positive by the size of their amplification products by multiplex PCR. Although these

TABLE 1. Primers used in this study

Gene	Primer	Sequence (5′→3′)	Size of PCR-amplified product (bp)	Reference or source
qnrA	qnrAF	ATTTCTCACGCCAGGATTTG	516	22
	qnrAR	GATCGGCAAAGGTTAGGTCA		
qnrB	qnrBF	GATCGTGAAAGCCAGAAAGG	476	This study
	qnrBR2	ATGAGCAACGATGCCTGGTA		
qnrC	qnrCF	GGGTTGTACATTTATTGAATCG	307	This study
	qnrCR	CACCTACCCATTTATTTTCA		
qnrS	qnrSmF	GCAAGTTCATTGAACAGGGT	428	2
	qnrSmR	TCTAAACCGTCGAGTTCGGCG		
aac(6′)-Ib	aacIbF	TTGCGATGCTCTATGAGTGGCTA	482	17
	aacIbR	CTCGAATGCCTGGCGTGTTT		
qepA	qepAF	AACTGCTTGAGCCCGTAGAT	596	This study
	qepAR	GTCTACGCCATGGACCTCAC		
gyrA	gyrAWF	AAATCTGCCCGTGTCGTTGGT	344	25
	gyrAWR	GCCATACCTACGGCGATACC		
parC	parCWF	CTGAATGCCAGCGCCAAATT	168	25
	parCWR	GCGAACGATTTCGGATCGTC		

results were reproducible, only 37 (8%) were confirmed to have at least one of six PMQR genes (Table 2). PMQR genes were detected in 13 (5%) of 261 *E. coli* isolates, 13 (10%) of 135 *K. pneumoniae* isolates, and 11 (17%) of 65 *E. cloacae* isolates. Isolates harboring *qnrB* and *qnrS* numbered 22 and 4, respectively, but no isolates were positive for *qnrA* or *qnrC*. Fifty-one isolates (11%) were positive for *aac(6′)-Ib*, of which 10 (2% of the total) carried the *cr* variant. *qepA* was present in only one isolates (0.2%). Overall, *qnrB* was the most prevalent PMQR gene (22/461 [4.8%]). *qnr* genes were detected most frequently in *E. cloacae*, followed by *K. pneumoniae*, and lastly *E. coli*, the reverse of the order for *aac(6′)-Ib-cr* prevalence.

Most *qnrB* genes (16/22) were of the *qnrB4* or *qnrB10* variant, which were present as early as 1998. Two other alleles, *qnrB2* and *qnrB5*, were detected in five isolates and one isolate after the year 2000, respectively. Among *qnr* producers, *qnrB2*, *qnrB4*, and *qnrB5* were found in *E. coli*, *qnrB2*, *qnrB4*, and *qnrB10* in *K. pneumoniae*, and *qnrB4* and *qnrB10* in *E. cloacae*.

qnrB genes were found as early as 1998, but *qnrS*, *aac(6′)-Ib-cr*, and *qepA* genes emerged subsequently after the year 2000. PMQR genes tended to be detected more frequently overall after 2000 than in the previous period studied ($P = 0.25$) (Table 3), though there was significant change in these genes in *E. coli* isolates ($P = 0.012$). The overall prevalence of PMQR genes showed an increasing trend over time ($P = 0.19$), and there was also a significant increase in rates of ciprofloxacin resistance over time ($P < 0.001$) (Fig. 1). Increasing ciprofloxacin resistance rates in *Enterobacteriaceae* tended to be correlated with increased prevalence of PMQR genes (Spearman's correlation coefficient = 0.657; $P = 0.16$). In addition, fluoroquinolone use increased from 27.8 (DDD per 1,000 patient days) in 2001 to 74.6 in 2006 ($P < 0.0001$).

Among the total isolates, those that were PMQR positive had significantly higher ciprofloxacin resistance and MDR rates ($P = 0.005$ and $P < 0.001$, respectively) (Table 3). In *E. coli*, however, the possession of PMQR genes was not associated with an increase in ciprofloxacin resistance or MDR rates. There was a trend for increasing ciprofloxacin resistance by species and by PMQR gene, but in *K. pneumoniae*, ciprofloxacin resistance and MDR rates were significantly associated with the presence of PMQR genes; 14 of the 37 isolates harboring PMQR genes were ciprofloxacin resistant by CLSI criteria. That 23 of the 37 isolates harboring PMQR genes were ciprofloxacin susceptible by CLSI criteria highlights the ability of these genes to circulate widely and, because of their limited reduction in susceptibility, to go undetected by routine susceptibility testing in the clinical microbiology laboratory. The MICs of ciprofloxacin for 37 PMQR gene-positive strains ranged from 0.008 to >32 μg/ml (median, 0.38 μg/ml).

We determined the mutations in the QRDRs of *gyrA* and *parC* for 126 strains, including all of the PMQR-positive strains and a sample of 89 PMQR-negative strains (49 that are ciprofloxacin susceptible and 40 that are ciprofloxacin resistant) randomly chosen from the three 2-year periods (26 from 1998 to 1999, 31 from 2000 to 2001, and 32 from 2005 to 2006). Substitutions at codons 83 and/or 87 in the *gyrA* gene were detected in 46% (58/126) of the strains, but no substitution was found at codon 81, 82, 84, or 106. Among the 58 strains with *gyrA* mutations, 48 had additional mutations at codons 80 and/or 84 in the *parC* gene. There were no strains with a *parC* QRDR mutation alone. The prevalence of amino acid substitutions in the QRDR of *gyrA* and/or *parC* increased significantly over time among PMQR-positive strains ($P = 0.012$), whereas it was stable for PMQR-negative strains (Table 4). The MICs of ciprofloxacin for 58 strains with the mutations in the QRDR of *gyrA* with or without *parC* ranged from 0.016 to >32 μg/ml (median, 4 μg/ml).

DISCUSSION

The present study yielded a prevalence of 8.0% for PMQR genes among 461 consecutive isolates of *E. coli*, *K. pneumoniae*, and *E. cloacae* collected from 1998 to 2001 and from 2005 to 2006 in a tertiary hospital in the Republic of Korea. Although 65 were repeatedly positive by multiplex PCR, 28 initially *qnr*-positive strains were found to be false positive by sequencing. The reason why nonspecific bands were generated by multiplex PCR, even if not by monoplex PCR under the

TABLE 2. Prevalence of six PMQR determinants

Year	Species	Total no. of isolates	No. of isolates positive for:						No. of isolates with any PMQR gene (%)
			qnrA	*qnrB*	*qnrC*	*qnrS*	*aac(6′)-Ib*	*aac(6′)-Ib-cr*	*qepA*	
1998	*E. coli*	36					1			0
	K. pneumoniae	22		2			4			2
	E. cloacae	12		3			3			3
	Total	70		5			8	0		5 (7.1)
1999	*E. coli*	40								0
	K. pneumoniae	18		1			2			1
	E. cloacae	10		2			4			2
	Total	68		3			6	0		3 (4.4)
2000	*E. coli*	40		1			4	2		3
	K. pneumoniae	21					2			0
	E. cloacae	12		1			5			1
	Total	73		2			11	2		4 (5.5)
2001	*E. coli*	35				1	4	1		2
	K. pneumoniae	18					2	2		2
	E. cloacae	10		3			3			3
	Total	63		3		1	9	3		7 (11.1)
2005	*E. coli*	54					2	1	1	2
	K. pneumoniae	29		4			5	1		5
	E. cloacae	11		2			4			2
	Total	94		6			11	2	1	9 (9.6)
2006	*E. coli*	56		3		1	4	2		6
	K. pneumoniae	27		0		2	2	1		3
	E. cloacae	10		0		0				0
	Total	93		3		3	6	3		9 (9.7)
Total	*E. coli*	261		4		2	15	6	1	13 (5.0)
	K. pneumoniae	135		7		2	27	4		13 (9.6)
	E. cloacae	65		11			19	0		11 (16.9)
	Total	461		22		4	51	10	1	37 (8.0)

same condition, is not yet understood. Therefore, this method could be used only for screening, and further monoplex PCR for each *qnr* gene or direct sequencing of the PCR product would be warranted for confirmation.

Although the prevalence of each PMQR gene varied by species, the overall prevalence was higher in *E. cloacae* and *K. pneumoniae* than in *E. coli*, as noted by other authors (10, 18, 22, 31). The most frequent PMQR gene was *qnrB*, as in other studies (2, 13, 22, 27, 30, 31). Whereas the *qnr* genes predominated in *K. pneumoniae* and *E. cloacae*, *aac(6′)-Ib-cr* was the most prevalent gene in *E. coli*. These differences are in accord with previous observations (17), but the cause is not yet understood. *qepA*, which was recently found in Japan and Belgium (19, 32), has been rarely present in the Republic of Korea, as has also been true in Japan and Brazil (13, 33). Since *qnrB*-positive isolates were identified as early as 1998, some of the PMQR genes have been present for at least a decade in *K. pneumoniae* and *E. cloacae*. Furthermore both the types of PMQR genes and the varieties of *qnrB* alleles diversified over time.

PMQR-positive strains were significantly more frequently ciprofloxacin resistant than were PMQR-negative strains (2.7-fold), with the dominant difference found in *K. pneumoniae* (9.6-fold). In addition, PMQR-positive *K. pneumoniae* and *E. cloacae* isolates had significantly higher MDR rates (17- to 28-fold) than did PMQR-negative isolates. Notably, *qnrB* accounted for 75% (18/24) of the PMQR genes detected in *K. pneumoniae* and *E. cloacae*. This result suggests an association

TABLE 3. Characteristics of PMQR-positive and PMQR-negative isolates

Characteristic	No. of isolates with characteristic/total no. of isolates with indicated PMQR result		Odds ratio (95% confidence interval)	*P* value
	Positive	Negative		
Isolation after 2000 vs before 2000[a]				
E. coli	13/185 vs 0/76	172/185 vs 76/76	1.44 (1.33–1.57)	0.012
K. pneumoniae	10/95 vs 3/40	85/95 vs 37/40	1.45 (0.38–5.58)	0.75
E. cloacae	6/43 vs 5/22	37/43 vs 17/22	0.55 (0.15–2.06)	0.48
Total	29/323 vs 8/138	294/323 vs 130/138	1.60 (0.68–3.92)	0.25
Ciprofloxacin resistance				
By species:				
E. coli	4/13	59/248	1.17 (0.36–3.80)	0.76
K. pneumoniae	6/13	10/122	9.60 (2.70–34.1)	0.001
E. cloacae	4/11	9/54	2.86 (0.69–11.8)	0.21
By PMQR gene:				
qnrB	7/22	78/424[b]	2.07 (0.82–5.25)	0.158
Any *qnr* gene	9/26	78/424[b]	2.35 (1.01–5.46)	0.068
aac(6′)-Ib-cr	4/10	78/424[b]	2.96 (0.82–10.7)	0.10
Total	14/37	78/424	2.70 (1.25–5.77)	0.005
MDR[c]	21/37	74/424	6.21 (2.94–13.2)	<0.001
E. coli	2/13	46/248	0.80 (0.17–3.73)	1.00
K. pneumoniae	9/13	14/122	17.4 (4.72–63.9)	<0.001
E. cloacae	10/11	14/54	28.6 (3.35–243.8)	<0.001

[a] Values for this characteric represent the number of strains isolated after 2000 versus the number of strains isolated before 2000.
[b] Only the number of ciprofloxacin-resistant and -susceptible strains without PMQR genes was included for comparison.
[c] MDR is defined as resistance to at least three different classes of antimicrobials.

between *qnrB* and other antibiotic resistance genes, which has also been noted by other investigators (3, 9, 15, 28). However, this linkage was not seen in *E. coli* isolates harboring the PMQR genes included in the study. In addition, the *qepA*-harboring isolate did not demonstrate the aminoglycoside resistance phenotype of *rmtB*, a gene closely linked to *qepA* in previous reports (19, 33). Therefore, further genetic analysis of the *qepA* plasmid(s) seems to be warranted.

There was an increasing trend in the number of PMQR-positive strains in the periods before and after 2000. In addition, there was a significant increase in the rates of ciprofloxacin resistance over the same time. Therefore, the increasing prevalence of PMQR genes may have been an important driving force for selection of quinolone resistance, although a causal link cannot be proven by this relationship alone. The demonstration in vitro that the low-level resistance conferred by *qnrA* may not only allow bacteria to survive in the presence of a quinolone but also substantially enhance the number of resistant mutants that can be selected from the population (8, 20, 24) supports the hypothesis that the increasing prevalence of the PMQR genes has contributed to the rise in resistance to fluoroquinolone in *Enterobacteriaceae*. Furthermore increases in selection pressure from the use of fluoroquinolones over the study period may have contributed both to the prevalence of the PMQR genes and to higher levels of ciprofloxacin resistance that these genes can facilitate.

TABLE 4. Trend in the prevalence of amino acid substitutions in the QRDR of *gyrA* and/or *parC* in 126 selected strains

Strain[a]	No. of isolates with mutation/total no. of isolates				*P* value
	1998–2006	1998–1999	2000–2001	2005–2006	
PMQR positive	12/37	0/8	3/11	9/18	0.012
CIP-R	9/14	0/3	2/2	7/9	
CIP-S	3/23	0/5	1/9	2/9	
PMQR negative	46/89	12/26	18/31	16/32	0.814
CIP-R	39/40	10/10	16/16	13/14	
CIP-S	7/49	2/16	2/15	3/18	
Total	58/126	12/34	21/42	25/50	0.214
CIP-R	48/54	10/13	18/18	20/23	
CIP-S	10/72	2/21	3/24	5/27	

[a] CIP-R, ciprofloxacin resistant; CIP-S, ciprofloxacin susceptible.

In order to investigate the roles of the QRDR mutations and the PMQR genes in contributing to higher levels of ciprofloxacin resistance over time, we determined mutations in the QRDR of *gyrA* and *parC* from PMQR-positive and -negative strains. Although the overall prevalence of the amino acid substitutions in these genes fluctuated around 50% in PMQR-negative strains, it increased significantly from 0% in 1998 to 1999 to 50% in 2005 to 2006 among PMQR-positive strains, especially in strains with a ciprofloxacin resistance phenotype (from 0% to 78%). The presence of Qnr proteins or Aac(6′)-Ib-cr is known to facilitate selection of resistance mutations in the presence of quinolone concentrations that would otherwise be lethal (8, 24). Thus, our data provide additional epidemiological support for the role of PMQR in promoting both

QRDR mutations in *gyrA* and *parC* and increased quinolone resistance in clinical settings as well.

This study has some limitations. Although we designed new, simple, and rapid multiplex PCR methods to detect all known PMQR genes, some of the new *qnr* variants, especially *qnrB8*, would be overlooked. Therefore, the prevalence of the PMQR genes reported here should be considered a minimum estimate. We did not amplify the QRDRs in *gyrB* and *parE*, because mutations in these regions have been substantially less frequently detected and confer lower levels of resistance relative to those conferred by *gyrA* or *parC* mutations (5). We did not include strains from the period of 2002 to 2004. Although the lack of data over this period and the relatively small number of PMQR-positive strains might decrease the statistical power to detect temporal correlations, there was nevertheless a significant increasing prevalence of PMQR genes over time and an association between increased prevalence of PMQR genes and increasing ciprofloxacin resistance. Finally we could not infer how much each PMQR gene or multiple genes contribute(s) to increase ciprofloxacin MIC in each species. For *E. coli*, *qnrA* transferred on a plasmid together with *aac(6′)-Ib-cr* conferred a ciprofloxacin MIC of 1.0 μg/ml (21), the breakpoint for ciprofloxacin susceptibility. The direct relationship of quinolone MIC and PMQR genes, however, has not been studied under defined genetic conditions in other species of *Enterobacteriaceae*.

In conclusion, the increasing frequency of quinolone resistance in *Enterobacteriaceae* was associated with an increasing prevalence and diversity of PMQR genes in consecutive samples of isolates and also an increasing prevalence of the QRDR mutations in PMQR-positive strains. These factors together with increasing use of fluoroquinolones created the opportunity for the emergence of highly quinolone-resistant clinical isolates associated with MDR that compromised therapeutic options in species that were initially highly susceptible to fluoroquinolones and would have been expected to have a low likelihood for the emergence of quinolone resistance.

ACKNOWLEDGMENTS

We thank Que Chi Truong-Bolduc, Yanpeng Ding, and Minghua Wang for technical advice and Hee-Chang Jang and Sae-Ick Joo for retrieving the isolates. We also express our gratitude to Minggui Wang and Patrice Courvalin for the gifts of control strains for *qnrC* and *qepA*, respectively.

This work was supported in part by grants R01AI057576 (to D.C.H.) and R01AI043312 (to G.A.J.) from the National Institutes of Health, U.S. Public Health Service.

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