



Characterization of genetic structures of the *QepA3* gene in clinical isolates of *Enterobacteriaceae*

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QepA is one of the genes that confer quinolone resistance in bacteria. The aim of this study was to analyze the genetic structures of plasmids that carry a *qepA3*, a recently discovered allele of *qepA* in *Enterobacteriaceae* clinical isolates. 656 non-redundant *Enterobacteriaceae* clinical isolates were screened for the *qepA3* gene and five isolates were identified to carry the gene. Plasmids were isolated from these isolates and were found to increase antibiotic resistance once the plasmids were transferred to *Escherichia coli*. These plasmids were subcloned and sequenced to analyze the genetic structures surrounding the *qepA3* gene. The results showed that the five plasmids had different genetic structures; two of the *qepA3*-containing isolates had either the *bla*_{CTX-M-14} or *bla*_{TEM-12} gene instead of the *bla*_{TEM-1} gene. The structures of both pKP3764 and pECL3786 have not been previously described. In comparison with pHPA, there were a number of changes in DNA sequences up- and down-stream of the *qepA3* gene. These findings provide better understanding of the genetic variations in *qepA3* and would be useful for diagnosis and control of quinolone resistance in clinical settings.

Keywords: plasmid-mediated quinolone resistance, *QepA3* gene, recombinant plasmid, genetic structure, gene variation

INTRODUCTION

According to past work (Cattoir et al., 2008), three mechanisms have been described for plasmid-mediated quinolone resistance: *qnr* determinants (Mammeri et al., 2005; Nordmann and Poirel, 2005; Robicsek et al., 2006; Wang et al., 2011), aminoglycoside acetyltransferase *aac(6)-Ib-cr* (Robicsek et al., 2006), and the *qepA* and *oqxAB* efflux pump genes, which confer decreased susceptibility to quinolones (Périchon et al., 2007; Yamane et al., 2007; Wong et al., 2015). The *qepA1* gene was first investigated in 2007 by two groups from Japan and Belgium (Périchon et al., 2007; Yamane et al., 2007). In 2008, the *qepA2* was discovered by a French research group (Cattoir et al., 2008). Currently, both the *qepA1* and *qepA2* genes have been reported worldwide (Liu et al., 2008; Park et al., 2009; Guillard et al., 2011; Ruiz et al., 2012; Chen et al., 2014). Recently,

we identified a new *qepA* allele *qepA3* from in a Chinese patient (GenBank with accession number JQ064560). Although the human *qepA* has a fairly low prevalence in Korea (Kim et al., 2009; Park et al., 2009), it is commonly found in *Enterobacteriaceae* isolates from food-producing animals in China (Liu et al., 2008; Ma et al., 2009; Chen et al., 2014). Therefore, more surveillance is needed for *Enterobacteriaceae* harboring the *qnr*, *aac(6′)-Ib-cr* and *qepA*.

In this study, we investigated the *qepA* gene in 656 *Enterobacteriaceae* isolates from hospitalized patients and only 13 isolates were found positive. This result confirm that *qepA* has low prevalence (1.98%) (0.91% for *qepA1*, 0.31% for *qepA2* and 0.76% for *qepA3*) in patients of our area. However, five isolates were determined to harbor novel *qepA3* structures. These structure were characterized to provide better understanding of the gene for potential management of plasmid-mediated antibiotic resistance.

MATERIALS AND METHODS

Bacterial Strains and Luria-Bertani (LB) Agar Plates

Enterobacteriaceae isolates EC3157, EC3587, CD4359, KP3764, and ECL3786 were identified using the Vitek 2 system (bioMérieux, France) according to the manufacturer's instructions. *Escherichia coli* isolates EC3157 and EC3587 were isolated from the blood and sputum samples of ICU inpatients, respectively. CD4359, an isolate of *Citrobacter koseri*, was obtained from the sputum of an inpatient in Infection Unit, while KP3764, an isolate of *Klebsiella pneumoniae*, was from a blood sample of an ICU inpatient. ECL3786, an isolate of *Enterobacter cloacae*, was from the chest wound secretions of a cardiothoracic surgical inpatient. *E. coli* JM109 was used as the host for cloning and an azide-resistant *E. coli* (strain J53) was used as the recipient strain for conjugation experiments. The LB1 agar medium for plasmid transformation contained 100 μ L of isopropyl β -D-1-thiogalactopyranoside (IPTG, 24 mg/mL), 200 μ L of 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal, 20 mg/mL) and 100 μ L of ampicillin (Amp, 100 mg/mL) in 100 mL medium. LB2 agar plates were used for conjugation experiments and were supplemented with sodium azide (150 μ g/mL) and ciprofloxacin (0.25 μ g/mL).

Susceptibility Testing

The minimum inhibitory concentrations (MICs) of eight antimicrobial agents (nalidixic acid, ofloxacin, ciprofloxacin, cefotaxime, ceftazidime, amikacin, and gentamicin) were determined using the MicroScan microdilution panel (Scott, USA) broth dilution method. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the controls. The results were interpreted according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2014).

Plasmid Isolation and Sequence Analysis

Bacterial plasmid DNA was extracted using a plasmid extraction kit (TaKaRa, Japan) according to the manufacturer's instructions.

PCR amplifications were performed using primers based on the pHPA (Table 1, Yamane et al., 2007). PCR was run for 3 min at 94°C followed by 30 cycles of 1 min of denaturing at 94°C and annealing at 56.9°C, with a final elongation of 10 min at 72°C on Life Veriti® PCR machine (Invitrogen, USA). The total reaction volume was 20 μ L containing 4 μ L 5X PCR buffer, 0.4 μ L of 10 mM dNTPs, 1 μ L each of 10 μ M primers and 0.2 μ L Polymerase, with nuclease-free water filled up to 20 μ L, and bacterial plasmids harboring the *qepA* gene as template. The amplicons were digested with *DraI* and *BamHI* (TaKaRa, Japan), ligated to linearized pMD19-T (TaKaRa, Japan), and transformed into *E. coli* JM109 competent cells. Plasmids DNA from Amp resistant colonies recovered on LB1 plate were sequenced.

Conjugation

The five isolates were conjugated with *E. coli* J53 as described previously (Wang et al., 2003). In the conjugation experiments, the isolates were used as donors and azide-resistant *E. coli* J53 as the recipient strain by filter mating. Transconjugants were selected on the LB2 agar plates. MICs for the donors, transconjugants, and recipients were also measured as described above.

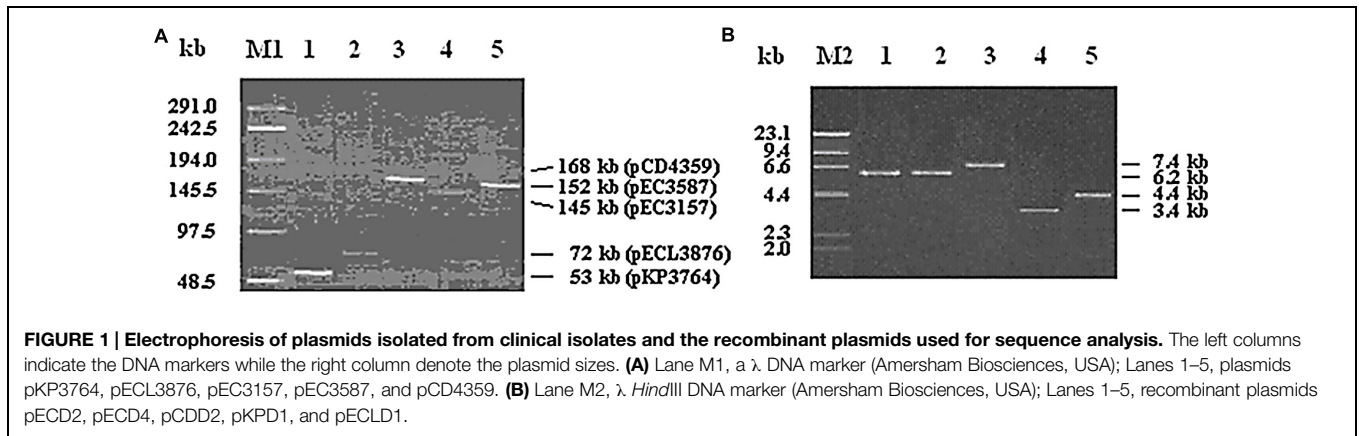
RESULTS

Plasmid Isolation and Characterization

Plasmids were isolated from the five *Enterobacteriaceae* isolates and separated on 0.7% agarose gel by gel electrophoresis. Results showed that plasmids pKP3764, pECL3876, pEC3157, pEC3587, and pCD4359 from isolates KP3764, pECL3876, EC3157, EC3587, and CD4359 were estimated to be about 53 kb, 72 kb, 145, 152, and 168 kb, respectively (Figure 1A).

TABLE 1 | Primers used for PCR and sequencing.

Primer	Sequence (5′-3′)	Reference
tnpR	F CGACACTGCCGATATGATCC	Park et al., 2009
	R CGGGCAACTACTGAGCTGATG	
TEM	F ATAAAATTCCTGAAGACGAAA	Lee et al., 2003
	R GACAGTTACCAATGCTTAATC	
rmtB	F CCCAACAGACCGTAGAGGC	Lee et al., 2006
	R CTCAAACCTCGGCGGGCAAGC	
qepA	F AGCAGCGCGCTGAATCCA	This study
	R CGAACCCAGTGGACATAA	
qepA (sequencing)	F AGC AGCGCGCTGAATCCA	This study
	R CTTCTGCCCCGAGTA TCG TG	
intl1	F GCCTTGCTGTTCTTCTACGG	Han et al., 2004
	R GATGCCTGCTTGTCTACGG	
tnpA	F GGCGGGATCTGCTTGTAGAG	Han et al., 2004
	R CTCCGGAGATGTCTGGCTTACT	
dfr2	F TTGGGCTTACCAGAGTATCAAGTT	Cattoir et al., 2008
	R GCTGTGGACGGTGCCGCATGATTTG	
CTX-M-14	F GAAAGAGAGTGAACGGATG	This study
	R ATTGGAAGGGTTCATCACC	



The *qepA*-containing sequences in the five strains were amplified, digested with *Dra*I and/or *Bam*HI and subcloned in pMD19-T to generate recombinant plasmids pECD1, pECD2, pECD3, pECD4, pCDD2, pKPD1, and pECLD1 (Figure 1B). The inserts in these plasmids were sequenced and analyzed for *qepA* and its flanking structures (GenBank accession numbers KR259130, KR259131, KR259132, KR259133, and KR259134, Supplementary Material) (Figure 2). The results showed that in addition to *qepA3*, which was present in all plasmids, the inserts from pEC3157 and pCD4359 contained the *bla*_{CTX-M-14} and *rmtB* genes; the inserts from pEC3587 had the *bla*_{TEM-12} gene; inserts from all plasmids except pCD4359 had the truncated *dfr2* gene; the inserts from pCD4359 contained the *bla*_{TEM-1} and *rmtB* genes; the insert from pKPD1 and pECLD1 contained truncated *dfr2* gene. No other resistant genes were found in these sequences.

Antibiotic Susceptibility

Through conjugation, the plasmids from the five isolates (donors) were transferred to *E. coli* J53 (recipients). The MIC values

for cefotaxime and ceftazidime in the transconjugants with the isolates EC3157, EC3587, and CD4359 were much higher than those of the recipient *E. coli* J53 (Table 2). Similarly, the MIC values for nalidixic acid, norfloxacin, ofloxacin, and ciprofloxacin in the transconjugants with the five isolates were generally higher than those of *E. coli* J53 (Table 2). Contrastingly, the MIC values nalidixic acid, ofloxacin, ciprofloxacin, amikacin, and gentamicin in the transconjugant involving the EC3157, EC3587, CD4357, and ECL3786 were generally higher than those of *E. coli* J53 (recipient) (Table 2). These results demonstrated that the antibiotic resistant genes are located in the plasmids.

DISCUSSION

Previous studies have shown that quinolone and fluoroquinolone resistance genes in *E. coli* and *Klebsiella* isolates are located on plasmids, which often carry other antimicrobial resistant genes, and can be transferred to other strains by conjugation or transformation (Ruiz et al., 2012). Since the discovery of

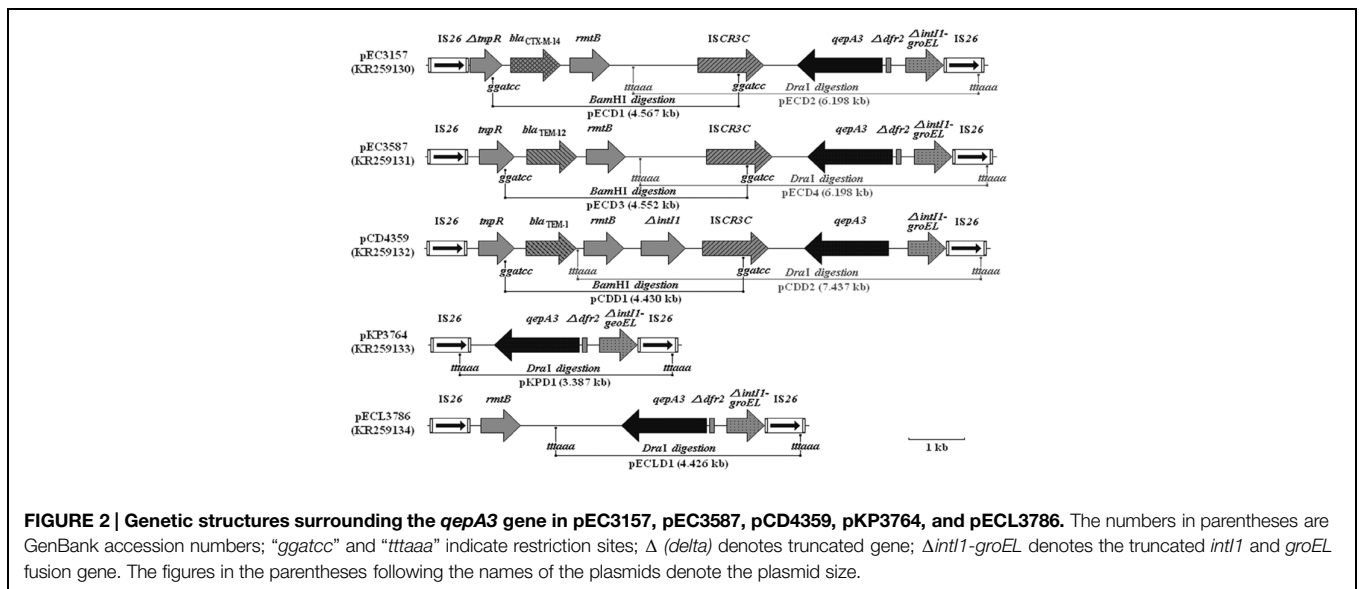


TABLE 2 | Minimum inhibitory concentrations of antimicrobial agents for donors, transconjugants, and their recipients.

Stain	MIC ($\mu\text{g}/\text{mL}$)							
	NAL	NOR	OFL	CIP	CTX	CAZ	AMK	GM
Donor								
EC3157	128	8	4	2	128	4	64	128
EC3587	128	16	4	4	32	4	128	>128
CD4359	128	16	4	4	32	2	64	128
KP3764	128	8	4	2	0.25	0.25	0.50	0.125
ECL3786	32	8	2	2	0.125	0.125	64	128
Transconjugant								
EC3157- <i>Escherichia coli</i> J53	8	2	1	0.50	8	1	16	32
EC3587- <i>E. coli</i> J53	16	4	1	1	4	1	32	64
CD4359- <i>E. coli</i> J53	16	4	1	1	4	1	16	64
KP3764- <i>E. coli</i> J53	16	2	1	0.50	0.125	0.125	0.50	0.063
ECL3786- <i>E. coli</i> J53	8	2	0.50	0.50	0.063	0.125	16	32
Recipient								
<i>E. coli</i> J53	4	0.25	0.032	0.016	0.063	0.25	0.50	0.063

NAL, nalidixic acid; OFL, ofloxacin; CIP, ciprofloxacin; CTX, cefotaxime; CAZ, ceftazidime; AMK, amikacin; GEN, gentamicin; NOR, norfloxacin.

qepA1 and *qepA2* in 2007 and 2008, the genetic environment and location of the *qepA* genes in the plasmid pHPA have both been well-established (Périchon et al., 2007; Yamane et al., 2007; Cattoir et al., 2008). In this study, five *Enterobacteriaceae* isolates carrying the *qepA3* and surrounding genes were investigated, and are found to have different genetic structures (Figure 2) surrounding *qepA3*.

The plasmid pCD4359 from isolate CD4359 showed a similar structure to pHPA, except for the *qepA* allele and a truncated *dfr2*, a gene closely related to vertebrate FGF-receptor. The plasmids pEC3587 and pEC3157 were different from pHPA, where *bla*_{TEM-1} in pHPA was replaced by *bla*_{TEM-12} or *bla*_{CTX-M-14}, respectively (Figure 2). *bla*_{CTX-M-14}, *bla*_{TEM-1}, and *bla*_{TEM-12} code β -lactamase and are important determinants of drug resistance. In addition, in pCD4359, there is an additional Δ *int11* downstream of the *rmtB* which confers high-level resistance to all aminoglycoside and missing Δ *dfr2*. The sequence between IS26 and *tnpR* was also truncated. Furthermore, *rmtB* or ISCR3C are not linked with *qepA3* directly in pKP3764 as reported previously (Périchon et al., 2007; Yamane et al., 2007; Cattoir et al., 2008; Kim et al., 2009; Rocha-Gracia et al., 2010; Cao et al., 2014).

In pEC3587, the *bla*_{TEM-12} gene took the place of *bla*_{TEM-1} and had a truncated *dfr2* gene as compared with pHPA. These results are also different from previous reports (Périchon et al., 2007; Yamane et al., 2007; Cattoir et al., 2008; Kim et al., 2009; Rocha-Gracia et al., 2010; Cao et al., 2014). Surprisingly, in pKP3764 there are three genes *qepA3*, truncated *dfr2*, and truncated *int11-groEL* fusion gene between the two IS26 insertions, while in pKP3764 and pECL3786, there are four genes: *rmtB*, *qepA3*, truncated *dfr2*, and truncated *int11-groEL* fusion genes. To date, these genetic structures have not been identified in previous studies (Kim et al., 2009; Rocha-Gracia et al., 2010; Cao et al., 2014). Moreover, pKP3764 is the only plasmid that does not contain the *rmtB* gene among the five plasmids studied in this study. According to a previous report, 58.3% (28/48) of *rmtB*-positive *E. coli* isolates harbored the

qepA gene (Grape et al., 2005). Whether or not these results suggest a strong link between *qepA* and *rmtB* remains to be investigated. In these five genetic structures (Figure 2), the *qepA3* gene and its downstream genes were nearly all identical except for the truncated *dfr2* deletion in pCD4359. After pEC3157 and pEC3587 were digested by the *DraI*, the size of both products were identical at approximate 6.2 kb. It should be noted that it is also possible that the ISCR3C is not a stable link between ISCR3C and the *qepA* gene.

QepA gene is a quinolone pump gene that confers resistance to nalidixic acid and norfloxacin. Stains carrying the gene may be resistant or sensitive to ofloxacin and ciprofloxacin with increased MIC. In the conjugation experiments, we found that *E. coli* cells with pEC3587 and pCD4359 were resistant to penicillins and aminoglycoside antibiotics drugs such as amikacin, gentamicin, gentamicin, and tobramycin. They were tolerant or sensitive to ofloxacin and ciprofloxacin, but the MICs were increased. These results indicate that there might be synergistic effect against antibiotics when the *qepA*, *bla*_{CTX-M 14}, and *rmtB* are present in the same plasmids, leading to multidrug resistance.

Taken together, our works suggest that there are sequence variations surrounding the *qepA3* even if a limited number of isolates are analyzed, and it is likely that more variations would exist that may impact the resistance profiles, and subsequently the clinical implications of the bacteria. More studies are needed to link these structure variation to resistance profiles and potential clinical outcomes.

CONCLUSION

The novel genetic structures surrounding the *qepA3* gene have been discovered in the isolates obtained from five patients in China although the prevalence for the *qepA* allele in hospital patients are low. One of the isolates is linked to the non-*rmtB*- or non- ISCR3C- producing genetic structure in the *qepA3* genetic

environment. Moreover, the *bla*_{CTX-M-14} or *bla*_{TEM-12} genes are found to be associated with the *qepA3* gene in these structures instead of the *bla*_{TEM-1} gene. These results provide new insight into the variation in genetic environments of the *qepA3* gene and would be useful for further investigation of the clinical implications in antibiotic resistance management.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01147>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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