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 blaCTX−M−14 or blaTEM−12 gene instead of the blaTEM−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 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 μg/mL of isopropyl-β-D-1-thiogalactopyranoside (IPTG, 24 mg/mL), 0.2 μL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 20 mg/mL), and 100 μL of ciprofloxacin (Amp, 100 mg/mL) in 100 mL medium. LB2 agar plates were used for conjugation experiments and were supplemented with sodium azide (150 μg/mL) and ciprofloxacin (0.25 μg/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 μL containing 4 μL 5X PCR buffer, 0.4 μL of 10 mM dNTPs, 1 μL each of 10 μM primers and 0.2 μL Polymerease, with nuclease-free water filled up to 20 μL, 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 JM110 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 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).

| Primer | Sequence (5'-3') | Reference |
|--------|-----------------|-----------|
| trpR   | F CGACACTGCCCATATGATCC | Park et al., 2009 |
| R CGGCCAATCTAGCGTGATG | |
| TEM    | F ATAAAATTCTGGAAGACGAAAA | Lee et al., 2003 |
| R GACGTTACCATGTTAACCTC | |
| rmtB   | F CCAACACAGACGATAGGCGGC | Lee et al., 2006 |
| R CTCAAACCTGGGCAGGCAAGGC | |
| qepA   | F AGCAGGCGGCGTAACTTCA | This study |
| R CGAACCCAGTGACATACAA | |
| qepA   | (sequencing) | This study |
| R AGC AGCAGGCGTAACTTCA | |

| Primer | Sequence (5'-3') | Reference |
|--------|-----------------|-----------|
| int1   | R CTTCCTGGCAGGTA TCG TG | Han et al., 2004 |
| R GCTTGTCTGCTTCTACGG | |
| dfr2   | F TTGGGCTTCACAGAGATCAGTGG | Cattoir et al., 2008 |
| R GTCTGGAGCTGCGCATGATTTGG | |
| CTX-M-14 | F GAAAGAVAGGCAACGCGAGTG | This study |
| R ATTGGAAGAGGTTCTACACC | |
The *qep A*-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 *qep A* and its flanking structures (GenBank accession numbers KR259130, KR259131, KR259132, KR259133, and KR259134, Supplementary Material) (*Figure 2*). The results showed that in addition to *qep A*, which was present in all plasmids, the inserts from *pEC3157* and *pCD4359* contained the *blaCTX−M−14* and *rmtB* genes; the inserts from *pEC3587* had the *blaTEM−12* gene; inserts from all plasmids except *pCD4359* had the truncated *dfr2* gene; the inserts from *pCD4539* contained the *blaTEM−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 (µg/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| 0.50| 0.125|     |
| ECL3786          | 32          | 8   | 2   | 2   | 0.125| 0.125| 64  |     |     |
| **Recipient**    |             |     |     |     |     |     |     |     |     |
| E. coli J53      | 4           | 0.25| 0.032| 0.016| 0.063| 0.25| 0.50| 0.063|     |

| Stain | MIC (µg/mL) | NAL | NOR | OFL | CIP | CTX | CAZ | AMK | GM |
|-------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|
| **Transconjugant** |             |     |     |     |     |     |     |     |     |
| EC3157- Escherichia coli J53 | 8 | 2 | 1 | 0.50 | 8 | 1 | 16 | 32 |     |
| EC3587- E. coli J53 | 16 | 4 | 1 | 1 | 4 | 1 | 32 | 64 |     |
| CD4359- E. coli J53 | 16 | 4 | 1 | 1 | 4 | 1 | 16 | 64 |     |
| KP3764- E. coli J53 | 16 | 2 | 1 | 0.50 | 0.125 | 0.125 | 0.50 | 0.063|     |
| ECL3786- E. coli J53 | 8 | 2 | 0.50 | 0.50 | 0.063 | 0.125 | 16 | 12 | 32 |

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

The genetic environments and location of the qepA3 gene

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; Kim et al., 2009; Rocha-Gracia et al., 2010; Cao et al., 2014). Surprisingly, 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 blaTEM-1 was replaced by blaTEM-12 or blaCTX-M-14, respectively (Figure 2). blaCTX-M-14blaTEM-1, and blaTEM-12 code β-lactamase and are important determinants of drug resistance. In addition, in pCD3589, there is an additional ΔintI1 downstream of the rmtB which confers high-level resistance to all aminoglycosides and missing Δdfr2. The sequence between IS26 and inpR 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 blaTEM-12 gene took the place of blaTEM-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 intI1-groEL fusion gene between the two IS26 insertions, while in pKP3764 and pECL3786, therefore are four genes: rmtB, qepA3, truncated dfr2, and truncated intI1-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 pCD3589. After pEC3157 and pEC3587 were digested by the DraI, the size of both products were identical at approx. 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 pCD3589 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, blaCTX-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 blaCTX-M-14 or blaTEM-12 genes are found to be associated with the qepA3 gene in these structures instead of the blaTEM-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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