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Original article

Characterization of plasmid-mediated quinolone resistance determinants in *Klebsiella pneumoniae* and *Escherichia coli* from Tokai, Japan

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ABSTRACT

The spread of plasmid-mediated quinolone resistance (PMQR) determinants was evaluated in 150 ceftazidime or cefotaxime-resistant clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from Tokai, Japan between 2008 and 2011. In this study, *qnrB*, *qnrS*, and *aac(6′)-Ib-cr* genes were detected in 12 (50.0%), 4 (16.7%), and 1 (4.2%) of 24 *K. pneumoniae* isolates, respectively, while *qnrA*, *aac(6′)-Ib-cr*, and *qepA* genes were detected in 1 (0.8%), 11 (8.7%), and 2 (1.6%) of 126 *E. coli* isolates, respectively. *qnr* genes were mainly found in *K. pneumoniae* (66.7%) and to a lesser extent in *E. coli* (0.8%). We determined the genetic environment of the *qnrB4* gene in 24.6 kb class 1 integron structure, including *aar-2*, *cmlA*, *blaOXA-10*, *aadA1*, *qacEdelta1*, *sul1*, and *blaDHA-1*. In a time-kill assay, introduction of the *qnrB4* or *qnrS1* plasmid to the recipient *E. coli* strain decreased the bactericidal activities of fluoroquinolones such as ciprofloxacin, levofloxacin, and pazufloxacin.

1. Introduction

Since the introduction of fluoroquinolones at a market, fluoroquinolone resistance among *Enterobacteriaceae* has been observed mainly due to chromosomal mutations in DNA gyrase or topoisomerase IV [1]. In 1998, the first plasmid-mediated gene exhibiting fluoroquinolone resistance, currently named *qnrA1*, was identified [1]. This gene encoded the protein protecting DNA gyrase and topoisomerase IV from fluoroquinolones. Seven *qnrA*, 74 *qnrB*, 9 *qnrS*, 1 *qnrC*, 2 *qnrD*, and 6 *qnrVC* genes have been reported as of April 2014 (http://lahey.org/qnrStudies). Moreover, *aac(6′)-Ib-cr*, a variant aminoglycoside acetyl transferase capable of reducing ciprofloxacin activity, and *qepA*, an efflux pump, were subsequently identified as plasmid-mediated quinolone resistance (PMQR) genes [1]. While the prevalence of PMQR genes has been investigated worldwide, we have limited information about the prevalence of PMQR genes in Japan, in which the *qnrS* gene was the first to be globally identified in 2003 [1–5].

The cloning and expression of *qnrA*, *qnrB*, and *qnrS* in *Escherichia coli* J53 strain produced an approximately 32-fold increase in the MICs of ciprofloxacin [1]. Bactericidal activity is known to be another index by which the effect of a resistance gene can be assessed. It has been reported that the introduction of the *qnrA1*, *qnrB19*, or *qnrS1* plasmid modified the bactericidal activities of ciprofloxacin in a time-kill assay [6]. On the other hand, the bactericidal activities of other fluoroquinolones against a transformant carrying a *qnrB or qnrS* plasmid have not been investigated. In addition, changes in the bactericidal rate of fluoroquinolones by the introduction of a *qnr* plasmid have not been evaluated quantitatively.

The aim of this study was to investigate the prevalence of PMQR genes in Tokai, Japan. Moreover, we determined the class 1 integron sequence including the *qnrB4* gene, which was the most prevalent PMQR in Tokai, Japan. Furthermore, we evaluate the effect of plasmids carrying *qnrB4* or *qnrS1* on bactericidal activity of fluoroquinolones such as ciprofloxacin, levofloxacin, and pazufloxacin.

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2. Materials and methods

2.1. Bacterial isolates

A total of 150 *Enterobacteriaceae* clinical isolates were collected by seven hospitals in Aichi and Gifu prefecture between 2008 and 2011 with MICs of ceftazidime ≥8 μg/ml or cefotaxime ≥2 μg/ml. They included 24 *Klebsiella pneumoniae* and 126 *E. coli*. These isolates were recovered from the following sources: urine (44.7%), sputum (16.7%), blood samples (9.3%), pus (5.3%), and other sources including intratracheal aspirate, bile, vaginal secretions, and drain tip.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the broth microdilution method using Clinical and Laboratory Standards Institute (CLSI)-specified procedures, control strains, and interpretive criteria [7,8]. The following antimicrobial agents were tested: ciprofloxacin (CPFX), levofloxacin (LVFX), pazufloxacin (PZFX), piperacillin (PIPC), amoxicillin-clavulanic acid (AMC), ceftazidime (CAZ), cefotaxime (CTX), flomoxef (FMOX), imipenem (IPM), and meropenem (MEPM).

2.3. Detection of antimicrobial resistance genes

qnrA, qnrB, qnrS, qnrC, qnrD, aac(6′)-Ib, and *qepA* genes were screened by multiplex (*qnrA*, *qnrB,* and *qnrS*) or simplex (the others) PCR [9–11]. The subtypes of *qnrA*, *qnrB,* and *qnrS* as well as the variant *aac(6′)-Ib-cr* were confirmed by full-length sequencing [11,12]. Subsequently, the presence of ESBL genes (TEM, SHV, and CTX-M type), pAmpC genes (MOX, CIT, DHA, ACC, EBC, and FOX-type) were determined by PCR and sequencing using specific primers (Table 1) [13–18]. Mutations in the *gyrA* and *parC* genes were analyzed by amplification and sequencing of the quinolone resistance-determining region using specific primers for *K. pneumoniae* and *E. coli* (Table 1) [10].

2.4. Plasmid isolation and transformation

Plasmids from *qnr*-positive bacterial isolates were purified using the QIAGEN Plasmid Maxi Kit (QIAGEN Science Inc., MD, USA). The transformation of *qnr* plasmids was performed using ElectroMAX DH10B cells (Life Technologies, CA, USA) according to the manufacturer's instructions. Transformants of *qnr* plasmids were selected on Luria–Bertani (LB, Becton, Dickinson and Company, NJ, USA) agar plates containing ciprofloxacin (0.01 μg/ml). The transformants from the *K. pneumoniae* Y881 strain and *K. pneumoniae* Y889 strain were designated as TfKpY881 and TfKpY889, respectively.

2.5. Sequence analysis of plasmid carrying the qnrB4 gene

The plasmid carrying the *qnrB4* gene was extracted from the transformant from the *K. pneumoniae* Y881 strain (pTfKpY881) using the QIAGEN Plasmid Maxi Kit, and shotgun sequenced using Illumina HiSeq at Hokkaido System Science Co., Ltd. (Sapporo, Japan). Paired-end reads of 100 bp were assembled using Velvet.

2.6. Time-kill assay

The time-kill assay was performed in triplicate for three fluoroquinolones (ciprofloxacin, levofloxacin, and pazufloxacin) against TfKpY881, TfKpY889 (the transformant carrying the *qnrS1* plasmid) and recipient *E. coli* DH10B. Three fluoroquinolones were evaluated at 1/4×, 1/2×, 1×, 2×, and 4× their respective MICs. Test strains were inoculated at approximately 10^7 CFU into 100 μl of Mueller-Hinton broth containing each drug and incubated at 35 °C. Aliquots were removed at 0, 1, 2, 4, and 8 h postinoculation and plated on Mueller-Hinton agar for CFU counts [19]. Colony counts were determined by averaging counts of three samples per group. The time to 3-$\log_{10}$ CFU/ml reduction ($T_{99.9\%}$) was calculated from two neighboring points, of which viable cells were over and below the value calculated as CFU counts at 0 h minus 3-$\log_{10}$ CFU/ml using the FORECAST function of Microsoft Office Excel 2003.

2.7. Statistical analysis

We compared the prevalence of *qnr* genes between *K. pneumoniae* and *E. coli* by the Fisher's exact test statistics using SAS release 9.2 (SAS Institute Japan, Tokyo, Japan). Statistical analysis was performed by parametric Dunnett's test for multiple comparisons between the results for the recipient strain *E. coli* DH10B and the transformants carrying the *qnr* plasmids in the same experimental conditions (agent, concentration, and sampling time) in time-kill assay.

3. Results

3.1. Prevalence of PMQR determinants in Enterobacteriaceae clinical isolates

PMQR genes were detected in 31 (20.7%) of 150 *Enterobacteriaceae* clinical isolates in Tokai, Japan between 2008 and 2011 (Table 2). *qnr* genes were detected in 17 (11.3%) of 150 isolates. The prevalence of *qnr* genes was 66.7% (16/24) in *K. pneumoniae*, and comprised 11 *qnrB4*, 1 *qnrB6*, and 4 *qnrS1*. The prevalence of the *qnr* gene was 0.8% (1/126) in *E. coli*, and the detected subtype was *qnrA1*. A significant difference ($P < 0.0001$) was detected in the prevalence of *qnr* genes between *K. pneumoniae* and *E. coli*. The *aac(6′)-Ib-cr* and *qepA* genes were detected in 8.0% (12/150; 11 *E. coli* and 1 *K. pneumoniae* isolates) and 1.3% (2/150; 2 *E. coli* isolates), respectively.

3.2. Profiles of qnr-positive clinical isolates and transformants

ESBL genes were detected in 7 (41.2%) among 17 *qnr*-positive isolates, comprising 4 *blaCTX-M-14*, 2 *blaSHV-12*, and 1 *blaSHV-2* (Table 3). *blaDHA-1* was detected in 11 (64.7%) of *qnr*-positive isolates, and the prevalence of *blaDHA-1* was 91.7% (11/12) in *qnrB*-positive *K. pneumoniae* isolates. Especially, *qnrB4* was significantly related to *blaDHA-1*, and all the 11 *qnrB4*-positive isolates were detected with *blaDHA-1* in five different hospitals. All *qnrS*-positive isolates possessed the ESBL genes, 3 *blaCTX-M-14* and 1 *blaSHV-2*. Transformation experiments were performed on all 17 *qnr*-positive isolates, and only five transformants were successfully generated. We confirmed the presence of PMQR genes by PCR and sequencing in all transformants such as TfKpY874, 879, 881, 890, and 889.

3.3. Genetic environment of qnrB4 gene

We determined the 24.6 kb contig sequence including the *qnrB4* gene in pTfKpY881. Analysis of the sequence indicated that the *qnrB4* gene embedded in an IS*CR1* containing mobile element integron complex class 1 (Fig. 1). The integron included seven resistance genes such as *aar-2*, *cmlA*, *blaOXA-10*, *aadA1*, *qacEdelta1*, *sul1*, and *blaDHA-1* as well as *qnrB4*. The sequence of integron region was nearly identical (>99% identity) to that of pCFI-2 from *Citrobacter freundii* (Genbank accession no. JN215524).

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