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Short communication

# Analysis of plasmid-mediated multidrug resistance in *Escherichia coli* and *Klebsiella oxytoca* isolates from clinical specimens in Japan

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ABSTRACT

This study investigated the relationship of plasmid-mediated quinolone resistance (PMQR) and aminoglycoside resistance among oxyimino-cephalosporin-resistant *Escherichia coli* ($n = 46$) and *Klebsiella oxytoca* ($n = 28$) clinical isolates in Japan. Seventy-three isolates appeared to produce an extended-spectrum β-lactamase (ESBL) and one *K. oxytoca* isolate produced IMP-1 metallo-β-lactamase (MBL). Polymerase chain reaction (PCR) and sequencing confirmed that eight CTX-M-9/SHV-12-producing isolates, one IMP-1-producing *K. oxytoca* isolate, and six ESBL-positive *E. coli* isolates respectively possessed PMQR genes *qnrA1*, *qnrB6*, and *aac(6′)-Ib-cr*. All *qnr*-positive isolates also carried either *aac(6′)-Ib* or *aac(6′)-IIc* aminoglycoside acetyltransferase genes. Resistance determinants to β-lactams, quinolones and aminoglycosides were co-transferred with a plasmid of ca. 140 kb. The *qnrA1* gene was located downstream of insertion sequence IS*CR1* in complex class 1 integrons. A novel *qnrA1*-carrying class 1 integron with the cassette arrangement *aac(6′)-IIc–aadA2* as well as a unique class 1 integron with $bla_{IMP-1}$–*aac(6′)-IIc* cassettes on the plasmid carrying *qnrB6* were found in *K. oxytoca* isolates. We describe the identification of *qnrB6* and *aac(6′)-Ib-cr* and the close association of *qnr* with *aac(6′)-Ib* and *aac(6′)-IIc* for the first time in clinical isolates producing ESBL or MBL in Japan.



## 1. Introduction

Quinolone resistance is usually caused by chromosomal mutations, however the plasmid-mediated quinolone resistance (PMQR) determinants QnrA, QnrB, QnrS and AAC(6′)-Ib-cr have been described [1]. Although these PMQR determinants result in low-level quinolone resistance, such reduced susceptibility is important because it facilitates the selection of mutants with higher-level resistance. In Japan, although *qnrS* was first found in *Shigella flexneri* [2] and *qnrA* was also described in Enterobacteriaceae clinical isolates [3,4], the presence of clinical strains harbouring *qnrB* or *aac(6′)-Ib-cr* has not been reported. Furthermore, a recent prevalence study of *qnr* and the *qepA* plasmid-mediated efflux pump gene suggested a low prevalence of *Escherichia coli* harbouring *qepA* or *qnr* in Japan [5].

Many studies have shown that most *qnrA*-positive enterobacterial isolates are associated with plasmid-mediated AmpC-type β-lactamases, extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) [1,3,4,6,7]. *qnrA* is often located in complex class 1 integrons between common region 1 (CR1), comprising orf513 recombinase and a second copy of the 3′ conserved segment (3′-CS2), together with other resistance gene cassettes such as trimethoprim and aminoglycoside resistance cassettes [1]. However, little has been reported on the genetic context of *qnr* in Japan.

The present study was conducted to investigate the genetic relationship of the PMQR genes *qnr* and *aac(6′)-Ib-cr* as well as the 6′-*N*-aminoglycoside acetyltransferase [AAC(6′)] genes *aac(6′)-Ib* and *aac(6′)-IIc* among oxyimino-cephalosporin-resistant *E. coli* and *Klebsiella oxytoca* clinical isolates in Japan and to determine the structure of class 1 integrons, including the insertion sequence CR1 (IS*CR1*) element associated with *qnr*.

## 2. Materials and methods

### 2.1. Bacterial strains

A total of 46 *E. coli* and 28 *K. oxytoca* clinical isolates resistant to one or more of cefotaxime, ceftazidime and aztreonam, collected at the University of Tokyo Hospital (Tokyo, Japan) between November 2005 and October 2006, were examined in this study.

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### 2.2. Susceptibility testing

ESBL and MBL production was confirmed by double-disk synergy tests using clavulanic acid and ethylene diamine tetra-acetic acid (EDTA) as β-lactamase inhibitors, respectively. Minimum inhibitory concentrations (MICs) of amikacin, aztreonam, cefazolin, cefoperazone, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, imipenem, levofloxacin and meropenem were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute [8]. Etest (AB BIODISK, Solna, Sweden) was used to detect low-level reduction in ciprofloxacin and levofloxacin susceptibility. Quality control for the MICs was performed using the reference strains *Staphylococcus aureus* ATCC 21293, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

### 2.3. Polymerase chain reaction (PCR) and sequencing

PCR amplification was performed with Premix *Taq* enzyme (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Both strands of the purified PCR fragments were sequenced with an ABI PRISM 3130 DNA sequencer (Applied Biosystems, Foster City, CA) and a similarity search was conducted using the BLAST program (DDBJ, Shizuoka, Japan).

### 2.4. Characterisation of β-lactamases

The $bla_{TEM}$, $bla_{SHV}$ and $bla_{CTX\text{-}M}$ genes were amplified using the published primers [9,10]. The presence of MBL genes $bla_{IMP}$ and $bla_{VIM}$ was tested with type-specific primers described previously [11]. For all *qnr*-positive isolates, the nucleotide sequences of the *bla* genes were determined by sequencing.

### 2.5. Detection of the plasmid-mediated quinolone resistance determinants and the AAC(6′) genes

Screening for *qnrA*, *qnrB* and *qnrS* was carried out by multiplex PCR amplification as described previously [12]. Sequences of *qnrA* and *qnrB* were determined by PCR sequencing with primers 5′-TTGATAAAGTTTTTCAGCAA and 5′-CTAATCCGGCAGCACTATTA for *qnrA1* and primers 5′-ATGACGCCATTACTGTAT and 5′-CTAACCAATCACCGCGAT for *qnrB6*, designed to amplify a 647-bp fragment and a 681-bp fragment, respectively. The presence of *aac(6′)-Ib* and *aac(6′)-Ib-cr* variant was determined by PCR sequencing using a common primer pair [13]. The primer pair used for detection of *aac(6′)-IIc* was 5′-CCAACAATGCCGCAATAGTT and 5′-ATGACCACTTCCCCTTGATT, amplifying a 573-bp fragment, designed in the present study.

### 2.6. Conjugation experiments and extraction of plasmids

Conjugation experiments were performed in Luria–Bertani broth with nine *qnr*-positive clinical isolates as donors and an *E. coli* C600 strain as recipient. Transconjugant clones were selected on Drigalski agar (BTB agar) plates containing 100 mg/L rifampicin and 4 mg/L cefotaxime (or 0.03 mg/L ciprofloxacin in the absence of transfer with cefotaxime). Plasmid DNA was extracted from donors and transconjugants using a NucleoBond® Xtra Midi (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and was subjected to electrophoresis on 0.7% (w/v) agarose (Takara Bio) gel with ethidium bromide at 50 V for 3 h. The size of transferred plasmids was estimated by adding up EcoRI and NotI restriction fragments.

### 2.7. Analysis of the genetic environment of qnr and class 1 integron structures

The genetic context of the *qnr* genes was investigated by PCR mapping and subsequent sequencing as described previously [14]. The content and order of the gene cassettes inserted between the 5′ conserved segment (5′-CS) and 3′-CS1 were determined by sequencing as described previously [15]. Finally, the regions between *intI1* in 5′-CS and a second copy of *qacEΔ1* in 3′-CS2 were sequenced using a primer walking strategy.

**Table 1**
Antibiotic resistance genes and susceptibility profiles in donors (*qnr*-positive isolates), the *Escherichia coli* recipient and transconjugants.

| Strain | Genotype of | | | Cassette array[a] | MIC (mg/L) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Qnr | β-Lactamase | AAC(6′) | | CIP | LVX | CFZ | CFP | CTX | CAZ | ATM | IPM | MEM | GEN | AMK |
| *Escherichia coli* (donors) | | | | | | | | | | | | | | | |
| E5 | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-Ib* | [*aadB–aadA2*] | >64 | 32 | >128 | >128 | 16 | <1 | 2 | ≤1 | ≤1 | >128 | 32 |
| E15 | *qnrA1* | $bla_{SHV\text{-}12}$ | *aac(6′)-Ib* | [*dfr16–aadA2*] | ≤0.5 | ≤0.5 | >128 | 32 | 32 | >64 | >64 | ≤1 | ≤1 | ≤2 | 8 |
| E18 | *qnrA1* | $bla_{SHV\text{-}12}$ | *aac(6′)-Ib* | [*dfr16–aadA2*] | ≤0.5 | ≤0.5 | >128 | 16 | 8 | >64 | >64 | ≤1 | ≤1 | ≤2 | 8 |
| *Klebsiella oxytoca* (donors) | | | | | | | | | | | | | | | |
| K5 | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-IIc* | [*aadB–aadA2*] | ≤0.5 | ≤0.5 | >128 | 64 | 8 | ≤1 | 4 | ≤1 | ≤1 | 8 | ≤2 |
| K7 | *qnrA1* | $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-Ib* | [*aadB–aadA2*] | ≤0.5 | ≤0.5 | >128 | >128 | 8 | 2 | >64 | ≤1 | ≤1 | 8 | 8 |
| K9 | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-IIc*[b] | [*aac(6′)-IIc–aadA2*] | ≤0.5 | 2 | >128 | 64 | 8 | 2 | 8 | ≤1 | ≤1 | 8 | ≤2 |
| K10 | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-IIc* | [*aadB–aadA2*] | ≤0.5 | 2 | >128 | 64 | 8 | ≤1 | 4 | ≤1 | ≤1 | 4 | ≤2 |
| K16 | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-IIc* | [*aadB–aadA2*] | ≤0.5 | 2 | >128 | 64 | 4 | ≤1 | 4 | ≤1 | ≤1 | 8 | ≤2 |
| K27 | *qnrB6* | $bla_{IMP\text{-}1}$[b] | *aac(6′)-IIc*[b] | [$bla_{IMP\text{-}1}$–*aac(6′)-IIc*] | 1 | 2 | >128 | >128 | 32 | >64 | ≤1 | 2 | 4 | ≤1 | ≤2 |
| *E. coli* C600 (recipient) | | | | | 0.016 | 0.032 | ≤2 | ≤8 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤2 |
| Transconjugants[c] | | | | | | | | | | | | | | | |
| E5TC-cip | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-Ib* | [*aadB–aadA2*] | 0.25 | 0.25 | N.T. | N.T. | 8 | ≤1 | ≤1 | ≤1 | ≤1 | 64 | 32 |
| E15TC-cip | *qnrA1* | $bla_{SHV\text{-}12}$ | *aac(6′)-Ib* | [*dfr16–aadA2*] | 0.25 | 0.25 | N.T. | N.T. | 32 | 32 | 64 | ≤1 | ≤1 | ≤1 | 16 |
| E18TC-cip | *qnrA1* | $bla_{SHV\text{-}12}$ | *aac(6′)-Ib* | [*dfr16–aadA2*] | 0.125 | 0.125 | N.T. | N.T. | 8 | 16 | >64 | ≤1 | ≤1 | ≤1 | 8 |
| K5TC-ctx | N.D. | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-IIc* | N.D. | 0.016 | 0.016 | >32 | >32 | 16 | ≤1 | >16 | ≤1 | ≤1 | 8 | ≤2 |
| K9TC-ctx | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-IIc*[b] | [*aac(6′)-IIc–aadA2*] | 0.25 | 0.25 | >32 | >32 | 32 | ≤1 | >16 | ≤1 | ≤1 | >8 | ≤2 |
| K10TC-ctx | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-IIc* | [*aadB–aadA2*] | 0.25 | 0.125 | >32 | >32 | 16 | ≤1 | 8 | ≤1 | ≤1 | 4 | ≤2 |
| K16TC-ctx | N.D. | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | N.D. | N.D. | 0.016 | 0.016 | >32 | >32 | 16 | ≤1 | >16 | ≤1 | ≤1 | ≤1 | ≤2 |
| K16TC-cip | *qnrA1* | $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ | *aac(6′)-IIc* | [*aadB–aadA2*] | 0.25 | 0.25 | N.T. | N.T. | 32 | ≤1 | 4 | ≤1 | ≤1 | 8 | 4 |
| K27TC-ctx | *qnrB6* | $bla_{IMP\text{-}1}$[b] | *aac(6′)-IIc*[b] | [$bla_{IMP\text{-}1}$–*aac(6′)-IIc*] | 0.125 | 0.25 | N.T. | N.T. | >16 | >64 | ≤1 | 2 | 4 | ≤1 | 2 |

MIC, minimum inhibitory concentration; CIP, ciprofloxacin; LVX, levofloxacin; CFZ, cefazolin; CFP, cefoperazone; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; GEN, gentamicin; AMK, amikacin; N.T., not tested; N.D., not detected.

[a] Gene cassette array in class 1 integrons.

[b] Genes carried as cassettes by the class 1 integrons.

[c] cip, transconjugants selected by ciprofloxacin; ctx, transconjugants selected by cefotaxime.