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Original article

# Comparison of the risk of acquiring *in vitro* resistance to doripenem and tazobactam/piperacillin by CTX-M-15-producing *Escherichia coli*

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ABSTRACT

To compare the risk of acquiring *in vitro* resistance between doripenem and tazobactam/piperacillin by CTX-M-15-producing *Escherichia coli*, the *in vitro* frequency of resistance was determined. Four strains carrying multiple β-lactamases such as $bla_{OXA-1}$ or $bla_{CTX-M-27}$ as well as $bla_{CTX-M-15}$ and $bla_{TEM-1}$ were used. No resistant colonies appeared on doripenem-containing plates, whereas resistant colonies were obtained from three of four test strains against tazobactam/piperacillin using agar plate containing 8- to 16-fold MIC of each drug. These three acquired tazobactam/piperacillin-resistant strains were not cross-resistant to doripenem, and they showed 1.9- to 3.1-fold higher piperacillin-hydrolysis activity compared to those of each parent strain. The change of each β-lactamase mRNA expression measured by real-time PCR varied among three resistant strains. One of three tazobactam/piperacillin-resistant strains with less susceptibility to ceftazidime overexpressed both $bla_{CTX-M-15}$ and $bla_{TEM-1}$, and the other two strains showed higher mRNA expression of either $bla_{TEM-1}$ or $bla_{OXA-1}$. These results demonstrate that multiple β-lactamases carried by CTX-M-15-producing *E. coli* contributed to the resistance to tazobactam/piperacillin. On the other hand, these resistant strains maintained susceptibility to doripenem. The risk of acquiring *in vitro* resistance to doripenem by CTX-M-15-producing *E. coli* seems to be lower than that to tazobactam/piperacillin.



## 1. Introduction

Extended-spectrum β-lactamases (ESBL)-producing Enterobacteriaceae have been rapidly increasing worldwide. Carbapenems, which are known to be stable to most of the ESBL, are the therapeutic option for treatment of infections caused by ESBL-producing strains [1]. β-Lactam/β-lactamase inhibitor combinations such as tazobactam/piperacillin have been also used, because tazobactam can effectively inhibit the hydrolysis activity of ESBL except for some inhibitor-resistant variants although piperacillin itself is a good substrate for ESBL as well as narrow-spectrum β-lactamases [1,2]. Some researchers have reported that carbapenems and β-lactam/β-lactamase inhibitor combinations showed similar clinical outcomes against bloodstream infections caused by ESBL-producing *Escherichia coli* [3]. However, according to other reports, the clinical effectiveness of β-lactam/β-lactamase inhibitor combinations compared with carbapenems is controversial in some cases such as high-inoculum infections [1,4,5].

Among ESBL-producing strains, CTX-M-15-producing *E. coli* classified as the highly virulent clone ST131 have been isolated worldwide from hospitals as well as community sites [6]. The plasmid encoding $bla_{CTX-M-15}$ often carries other antimicrobial resistance genes such as $bla_{TEM-1}$, $bla_{OXA-1}$, *tetA*, *aac*(6′)-*IB-cr* and *aac*(3)-*II*, leading to a multidrug resistant phenotype [7]. Therefore, it is important to evaluate the risk of acquiring resistance to well-used classes of antibiotics by clinically alarming pathogen. In this study, we compared the *in vitro* frequency of resistance between doripenem as carbapenem and tazobactam/piperacillin in CTX-M-15-producing *E. coli* including ST131 strains and analyzed the resistant mechanisms mainly focusing on β-lactamases.

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## 2. Materials and methods

### 2.1. Bacterial strains and characterization

The tested strains and MICs are listed in Table 1. They were isolated from Japanese medical facilities in 2004 and 2010. The β-lactamase genes were identified by using a single conventional PCR assay with the primer sets described in Table 2. The plasmidic AmpC genes were identified with the primer sets described previously [8]. Genomic DNA was extracted by heat lysis and PCR was conducted using GoTaq Master Mix (Promega) in C1000™ Thermal Cycler (Bio-Rad). Multilocus sequence typing (MLST) was performed according to the protocol and primer sets specified in the *E. coli* MLST web site (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

### 2.2. Antibiotic susceptibility testing

The following antibiotics were obtained from the indicated suppliers: piperacillin from LKT Laboratories, Inc.; tazobactam, clavulanic acid, sulbactam, ceftazidime, and amoxicillin from U. S. Pharmacopeia; ampicillin from Wako Pure Chemical Industries, Ltd.; doripenem, latamoxef (moxalactam) and flomoxef from Shionogi & Co., Ltd. The MIC was determined by broth microdilution using Mueller Hinton broth (MHB) (Difco) according to the Clinical Laboratory Standards Institute (CLSI) recommendation [9].

### 2.3. Determination of spontaneous mutation frequency

Bacterial inocula (approximately $10^7$ CFU/plate) were spread on Mueller Hinton agar (MHA) containing 8- or 16-fold MIC of each drug. Tazobactam was used at a fixed concentration of 4 μg/mL. The plates were incubated at 35 °C overnight and the number of resistant colonies was counted. Mutation frequency was determined as a ratio of the average number of resistant colonies on drug-containing plate per viable colony on drug-free plate.

### 2.4. Hydrolysis activity of β-lactamase

The bacterial strains were cultured in MHB at 35 °C. Bacterial cells were collected when the absorbance value was OD = 0.5–0.6 at 625 nm and washed with 20 mM sodium phosphate buffer (pH7.0). The cells were freeze-thawed and then disrupted on ice by two-times of sonication (BRANSON) with 25% amplitude for 30 s. The supernatant collected by centrifugation was used as the crude enzyme. After total protein concentration was adjusted to the same value among 6 tested samples (SR28632, SR28632-R, SR34176, SR34176-R, SR34199 and SR34199-R), the hydrolysis of piperacillin was detected by monitoring the change in the absorbance of piperacillin in 50 mM sodium phosphate buffer (pH 7.0) at 30 °C by U-3010 photospectrometer (Hitachi) at 232 nm. The hydrolysis rate of piperacillin was calculated by using coefficients $\Delta\varepsilon = -1640\ M^{-1}\ cm^{-1}$ [10]. The reaction was performed in a total volume of 0.5 mL. Three independent measurements were taken.

**Table 1**
Characteristics and MIC of CTX-M-15-producing *Escherichia coli*.

| Strain | Year | Characteristics[a] | MLST | MIC (μg/mL) | | | |
|---|---|---|---|---|---|---|---|
| | | | | DRPM | TZP | CAZ | CIP |
| SR21464 | 2004 | CTX-M-15, TEM-1 | 538 | 0.016 | 1 | 16 | ≤0.063 |
| SR28632 | 2004 | CTX-M-15, TEM-1, OXA-1 | 648 | 0.031 | 8 | 32 | >64 |
| SR34176 | 2010 | CTX-M-15, CTX-M-27, TEM-1 | 131 | 0.031 | 2 | 32 | 32 |
| SR34199 | 2010 | CTX-M-15, TEM-1 | 131 | 0.031 | 4 | 64 | 64 |

MLST, multi-locus sequence typing; DRPM, doripenem; TZP, tazobactam/piperacillin; CAZ, ceftazidime; CIP, ciprofloxacin.
Tazobactam was used at a fixed concentration of 4 μg/mL.
[a] These four strains were not encoding plasmidic AmpC.

**Table 2**
Nucleotide sequences of primers used in this study.

| Name | Sequence (5′–3′) | Reference |
|---|---|---|
| **Identification of ESBL genes** | | |
| CTX-M-1 group-F | CCGACATATGGTTAAAAAATCACTGCGTCAG | This study |
| CTX-M-1 group-R | CCGTGAATTCTTACAAACCGTTGGTGACGATTT | This study |
| TEM-F | CCGACATATGAGTATTCAACATTTTCGTGTC | This study |
| TEM-R | CCGTGAATTCTTACCAATGCTTAATCAGTGAGG | This study |
| OXA-1-F | CCGACATATGAAAAACACAATACATATCAACTT | This study |
| OXA-1-R | CCGTGAATTCTTATAAATTTAGTGTGTTTAGA ATGGT | This study |
| **Real-time PCR** | | |
| 16s rRNA-F | TCCATGAAGTCGGAATCGCTAG | [23] |
| 16s rRNA-R | CACTCCCATGGTGTGACGG | [23] |
| CTX-M-15-F | GGAATCTGACGCTGGGTAAA | This study |
| CTX-M-15-R | ACGGCTTTCTGCCTTAGGTT | This study |
| CTX-M-27-F | GCCTGCCGATCTGGTTAACTAC | This study |
| CTX-M-27-R | ATTCAGCGTAGGTTCAGTG | This study |
| TEM-1-F | GATAACACTGCGGCCAACTT | This study |
| TEM-1-R | TTGCCGGGAAGCTAGAGTAA | This study |
| OXA-1-F | AAGCATGGCTCGAAAGTAGC | This study |
| OXA-1-R | CGACCCCAAGTTTCCTGTAA | This study |
| **Analysis of the β-lactamase promoter mutations** | | |
| IS*Ecp1*-F | GCAGGTCTTTTTCTGCTCC | [24] |
| IS*Ecp1*-R | TTTCCGCAGCACCGTTTGC | [24] |

### 2.5. Relative quantification by real-time PCR

The bacterial strains were cultured in MHB at 35 °C. Bacterial cells were collected when the absorbance value was OD = 0.5–0.6 at 625 nm. Total RNA was purified using RNeasy mini kit (QIAGEN). DNase treatment was conducted with the RNase-Free DNase Set (QIAGEN). RT-PCR was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems) by 7500 Fast Real-Time PCR Systems (Applied Biosystems). The primers used in this study are listed in Table 2. The target gene mRNA expression was normalized against the 16S rRNA gene, then the mRNA expression fold change was calculated by ratio to parent. Three independent measurements were taken.

## 3. Results

### 3.1. Spontaneous mutation frequency

*In vitro* frequency of resistance to doripenem and tazobactam/piperacillin in four strains of CTX-M-15-producing *E. coli* was evaluated. No resistant colonies were obtained from doripenem-containing MHA plates. On the other hand, the resistant colonies appeared on tazobactam/piperacillin-containing plates from SR28632, SR34176 and SR34199, with $2.0 \times 10^{-7}$ to $4.8 \times 10^{-7}$ of the spontaneous mutation frequency (Table 3). The randomly selected three resistant colonies from the test plate of SR28632 showed similar MIC pattern against tazobactam/piperacillin and doripenem. Therefore, a representative one colony was randomly chosen from the three resistant colonies for further

**Table 3**
Spontaneous mutation frequencies against CTX-M-15-producing *Escherichia coli*.

| Test substance | SR21464 | SR28632 | SR34176 | SR34199 |
|---|---|---|---|---|
| DRPM | $<7.0 \times 10^{-8}$ | $<6.0 \times 10^{-8}$ | $<6.7 \times 10^{-8}$ | $<6.0 \times 10^{-8}$ |
| TZP | $<7.0 \times 10^{-8}$ | $4.2 \times 10^{-7}$ | $2.0 \times 10^{-7}$ | $4.8 \times 10^{-7}$ |

DRPM, doripenem; TZP, tazobactam/piperacillin.