Single or in Combination Antimicrobial Resistance Mechanisms of *Klebsiella pneumoniae* Contribute to Varied Susceptibility to Different Carbapenems

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Abstract

Resistance to carbapenems has been documented by the production of carbapenemase or the loss of porins combined with extended-spectrum β-lactamases or AmpC β-lactamases. However, no complete comparisons have been made regarding the contributions of each resistance mechanism towards carbapenem resistance. In this study, we genetically engineered mutants of *Klebsiella pneumoniae* with individual and combined resistance mechanisms, and then compared each resistance mechanism in response to ertapenem, imipenem, meropenem, doripenem and other antibiotics. Among the four studied carbapenems, ertapenem was the least active against the loss of porins, cephalosporinases and carbapenemases. In addition to the production of KPC-2 or NDM-1 alone, resistance to all four carbapenems could also be conferred by the loss of two major porins, OmpK35 and OmpK36, combined with CTX-M-15 or DHA-1 with its regulator AmpR. Because the loss of OmpK35/36 alone or the loss of a single porin combined with *bla*<sub>CTX-M-15</sub> or *bla*<sub>DHA-1</sub>-ampR expression was only sufficient for ertapenem resistance, our results suggest that carbapenemases other than ertapenem should still be effective against these strains and laboratory testing for non-susceptibility to other carbapenems should improve the accurate identification of these isolates.

Introduction

The increasing prevalence of extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* is a critical concern for scientists trying to develop treatments against bacterial infections. TEM-, SHV- and CTX-M-type ESBLs and CMY- and DHA-type AmpC β-lactamases are commonly found in multidrug-resistant *Enterobacteriaceae* [1-4]. Carbapenem has been recommended as an effective drug for these strains. However, carbapenem-resistant *Enterobacteriaceae* have recently been reported worldwide. In *Klebsiella pneumoniae*, numerous reports have confirmed carbapenem resistance by ESBLs or AmpC β-lactamases combined with the loss of outer membrane porins OmpK35 and/or OmpK36 [5-7], or by carbapenemases alone [8].

Currently, four carbapenems are used clinically (imipenem, meropenem, ertapenem and doripenem). The mechanisms of carbapenem resistance were mainly detected from clinical isolates with individual or combined genetic alterations. No comparisons have been made on all four carbapenems and on the contributions of each resistance mechanism towards carbapenem resistance. In the present study, we created mutants with individual or combined resistance mechanisms from a susceptible clinical *K. pneumoniae* isolate and studied each resistance mechanism in response to these four carbapenems.
Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *K. pneumoniae* clinical strains were isolated from different patients in Taiwan or America, and strain NVT2001 has been found to be resistant only to ampicillin [9]. The low-copy-number plasmid pACYC177 was used in the cloning experiments. *Escherichia coli* and *K. pneumoniae* strains were cultured at 37°C in Mueller-Hinton broth (MHB) or Luria-Bertani (LB) broth with the appropriate antibiotics.

Conjugation experiments and plasmid content

Direct transfer of the β-lactamase-carrying plasmids from *K. pneumoniae* clinical isolates to azide-resistant *E. coli* J53 was performed by filter conjugation [10]. Transconjugants were selected on LB agar plates containing sodium azide (100 μg/ml) for counterselection and cefotaxime (0.5–2 μg/ml) for selection of plasmid-encoded resistance. Because *E. coli* J53 does not retain the *bla*KPC2-carrying plasmids of clinical *K. pneumoniae* for any period of time [11], the *bla*KPC2-carrying plasmid was directly transferred to the *K. pneumoniae* strains NVT2001S, ΔompK35, ΔompK36 and ΔompK35/36 by filter conjugation [10]. Transconjugants were selected on LB agar plates containing streptomycin (500 μg/ml) for counterselection and cefotaxime (0.5–2 μg/ml) for selection of plasmid-encoded resistance. Resistance genes, including *blaCTX-M*, *blaSHV*, *blaTEM*, *blaOXA*, *blaOXY*, *blaMOX*, *blaLAT*, *blaBL*, *blaKPC* and *blaNDM*, were detected among the transconjugant plasmids using PCR with specific primers (Table S1) and then further confirmed by sequencing. All the sequence analyses were conducted using the NCBI database (http://www.ncbi.nlm.nih.gov/). Isolates carrying resistance genes that had previously been sequence-confirmed were used as positive controls in every PCR assay.

Cloning and strain construction

DNA fragments of the *blaCTX-M*, *blaSHV*, *blaTEM*, *blaOXA*, *ampR*, *blaKPC* and *blaNDM* genes with their flanking regions were amplified from transconjugants by PCR with specific primers (Table S1). The generated PCR fragments were digested with *PstI* or *BamHI* and then cloned into pACYC177. The resulting plasmids were then transformed into the *K. pneumoniae* strains NVT2001S, ΔompK35, ΔompK36 and ΔompK35/36 by electroporation. The recombinant bacteria were plated onto LB agar plates containing kanamycin (25 μg/ml), and the presence of the β-lactamase genes was confirmed by electrophoresis.

Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| **Strains** | | |
| *K. pneumoniae* | | |
| ZAT242 | Clinical isolate with plasmid pCT | This study |
| CGM#KL1 | Clinical isolate with plasmid pSH | This study |
| VGC263 | Clinical isolate with plasmid pDH | This study |
| KPC2010 | Clinical isolate with plasmid pKP | [11] |
| TAI2010 | Clinical isolate with plasmid pND | [27] |
| NVT2001S | Streptomycin-resistant isolate of clinical strain NVT2001 | [9] |
| ΔompK35 mutant | ompK35 deletion strain of NVT2001S; Sm² | [9] |
| ΔompK36 mutant | ompK36 deletion strain of NVT2001S; Sm² | [9] |
| ΔompK35/36 mutant | ompK35 and ompK36 deletion strain of NVT2001S; Sm² | [9] |
| *E. coli* | | |
| J53 | A recipient for conjugation experiment; Az² | [28] |
| **Plasmids** | | New England Biolabs |
| pACYC177 | Low-copy-number plasmid; Ap² Km² | |
| pCT | Plasmid from clinical isolate containing *blaCTX-M* | This study |
| pSH | Plasmid from clinical isolate containing *blaSHV* | This study |
| pDH | Plasmid from clinical isolate containing *blaOXA-1-ampR* | This study |
| pKP | Plasmid from clinical isolate containing *blaKPC* and *blaTEM* | [11] |
| pND | Plasmid from clinical isolate containing *blaNDM* | [27] |
| pCT177 | Fragment containing *blaCTX-M* and its flanking region cloned into pACYC177 | This study |
| pSH177 | Fragment containing *blaSHV* and its flanking region cloned into pACYC177 | This study |
| pDH177 | Fragment containing *blaOXA-1-ampR* and its flanking region cloned into pACYC177 | This study |
| pDH177 | Fragment containing *blaOXA-1-ampR* and its flanking region cloned into pACYC177 | This study |
| pPH177 | Fragment containing *blaDHA-1* and its flanking region cloned into pACYC177 | This study |
| pAMP177 | Fragment containing *ampR* and its flanking region cloned into pACYC177 | This study |
| pKP177 | Fragment containing *blaKPC* and its flanking region cloned into pACYC177 | This study |
| pND177 | Fragment containing *blaNDM* and its flanking region cloned into pACYC177 | This study |

²Ap², resistance to ampicillin; Az², resistance to azide; Km², resistance to kanamycin; Sm², resistance to streptomycin.
by PCR and sequencing. Direct transfer of the β-lactamase-carrying plasmids from E. coli J53 transconjugants to the K. pneumoniae strains NVT2001S, ΔompK35, ΔompK36 and ΔompK35/36 was performed by filter conjugation [10]. Transconjugants were selected on brilliant green containing inositol-nitrate-deoxycholate (BIND) plates containing ceftoxime (1 μg/ml) for the selection of plasmid-encoded resistance, and the growth of non-K. pneumoniae strains was effectively suppressed on BIND plates [12]. The presence of the β-lactamase genes was verified by PCR and sequencing.

Antimicrobial susceptibility test

Minimal inhibitory concentrations (MICs) of ertapenem, imipenem, meropenem and doripenem were determined using the E-test (Biodisk AB, Sweden). MICs of the other 15 antimicrobial agents were determined using a broth microdilution test according to the recommendations of the clinical and laboratory standards institute (CLSI) [13]. The following antimicrobial agents were used: aztreonam, ampicillin, piperacillin/tazobactam, cefazolin, cephalothin, cefoxitin, ceftriaxone, cefepime, cefodoxime, cefotaxime, cefotaxime/clavulanic acid, ceftazidime, cefazidime/clavulanic acid, cefepime, ciprofloxacin and gentamicin. All the results were interpreted according to the breakpoints established by the CLSI in 2013 [14].

Results

Strain construction and porin loss in antibiotic resistance

The β-lactamases detected in the clinical plasmids are shown in Table 1, while blaCTX-M-15, blaSHV-12, blaOXA-ampR, blaKPC-2 or blaNDM-1 was found in each of the five clinical plasmids. To evaluate the effect of these β-lactamases alone and in combination with porin loss on antimicrobial resistance, the clinical and recombinant plasmids were transferred into the NVT2001S strain and its porin-loss mutants. The loss of OmpK35 alone did not significantly influence the antibiotic resistance of the NVT2001S strain (Table 2). Conversely, the loss of OmpK36 alone conferred resistance to cefazolin, cephalothin and cefoxitin, and the loss of OmpK35/36 caused highly resistance against these cephalosporins (Table 2).

ESBLs with or without porin loss in antibiotic resistance

The production of CTX-M-15 or SHV-12 alone conferred resistance to aztreonam and many of the cephalosporins tested (Table 3). With the loss of OmpK36, the CTX-M-15 and SHV-12 strains showed resistance to aztreonam, piperacillin/tazobactam and all the cephalosporins tested, while some β-lactam/β-lactamase inhibitor combinations were still effective against these strains (Table 3).
Table 3. MICs of antibiotics against *K. pneumoniae* NVT2001S and its porin-loss mutants with extended-spectrum β-lactamase.

| Antibiotic | Minimal inhibitory concentration (µg/ml) |
|------------|----------------------------------------|
|            | NVT2001S | ΔompK35 | ΔompK36 | ΔompK35/36 |
| CTX-M-15 | pCt | LCP | pSH | LCP | pCT | LCP | pSH | LCP | pCT | LCP | pSH | LCP |
| Aztreonam | ≤4 | 128 | ≤16 | 16 | ≤128 | ≤16 | 128 | 32 | 128 | 16 | 64 | 32 | 16 |
| Ampicillin | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 |
| Piperacillin/TZB | 16 | ≤4 | ≤4 | 16 | 16 | ≤128 | ≤128 | ≤128 | ≤128 | ≤128 | ≤128 | ≤128 | ≤128 |
| Cefazolin | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 |
| Cephalothin | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 |
| Cefoxitin | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 | ≤4 |
| Ceftriaxone | ≥256 | ≥256 | ≥128 | ≥16 | ≥256 | ≥256 | ≥256 | ≥256 | ≥256 | ≥256 | ≥256 | ≥256 | ≥256 |
| Cefpodoxime | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 | ≥64 |
| Cefoxime | ≥128 | ≥128 | ≥32 | 16 | ≥128 | ≥128 | ≥128 | ≥128 | ≥128 | ≥128 | ≥128 | ≥128 | ≥128 |
| Cefotaxime/CLA | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 | ≤0.12 |
| Cefazedine | 16 | 64 | ≥256 | ≥256 | ≥128 | ≥128 | ≥256 | ≥256 | ≥256 | ≥256 | ≥256 | ≥256 | ≥256 |
| Cefazidime/CLA | 0.25 | 0.5 | 0.5 | 0.25 | 0.25 | 1 | 1 | 1 | 0.25 | 1 | 2 | 0.5 | 1 | 4 |
| Cefepine | ≥128 | ≥32 | ≤4 | 2 | 32 | 32 | 16 | 32 | 32 | 32 | 32 | 32 | 32 |
| Ciprofloxacin | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 | ≤1 |
| Gentamicin | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 | ≥32 |

**Table notes:**
- Tazobactam with a fixed concentration of 4 µg/ml; CLA, clavulanic acid with a fixed concentration of 4 µg/ml.
- Boldface numbers indicate a significant (≥4-fold) difference in the MICs of *K. pneumoniae* NVT2001S and its derived strains, while the underlined numbers were above the breakpoint of susceptibility established by CLSI in 2013 [14].
- MICs of antibiotics tested compared to that harboring the recombinant plasmid with *bla*<sub>ΔompK35/36</sub> alone. No significant (≥4-fold) differences in MICs were found between the AmpR strain and its parental strain (Table 4).
- The strains harboring the recombinant plasmid, the expression of *bla*<sub>ΔompK35/36</sub> could confer significantly (≥4-fold) higher MICs of many antibiotics tested compared to the expression of *bla*<sub>ΔompK35/36</sub> alone. No significant (≥4-fold) differences in MICs were found between the AmpR strain and its parental strain (Table 4).

**Antibiotic activity:**

**AmpC β-lactamases with or without porin loss in antibiotic resistance**

NVT2001S harboring the clinical plasmid with *bla*<sub>ΔompK35/36</sub> showed significantly (≥4-fold) higher MICs for several antibiotics tested compared to that harboring the recombinant plasmid with *bla*<sub>ΔompK35/36</sub>. This result indicates that other resistant genes should exist on the clinical plasmid (Table 4). For the strains harboring the recombinant plasmid, the expression of *bla*<sub>ΔompK35/36</sub> could confer significantly (≥4-fold) higher MICs of many antibiotics tested compared to the expression of *bla*<sub>ΔompK35/36</sub> alone. No significant (≥4-fold) differences in MICs were found between the AmpR strain and its parental strain (Tables 4). With the loss of OmpK35/36, the DHA-1-AmpR strains were highly resistant to aztreonam, piperacillin/tazobactam and all the cephalosporins tested except cefepime (Table 4).

**Carbapenemases with or without porin loss in antibiotic resistance**

In all the antibiotics tested, the KPC-2 strains showed susceptibility to ciprofloxacin and gentamicin. The NDM-1 strains showed susceptibility to aztreonam, ciprofloxacin and gentamicin, except gentamicin for the strains harboring the clinical plasmid with *bla*<sub>NDM-1</sub> (Table 5). The loss of porins did not increase the resistance of these strains to these antibiotics (Table 5).

**Single or in combination antimicrobial resistance mechanisms in carbapenem resistance**

The loss of OmpK35 or OmpK36 alone did not significantly (≥4-fold) influence carbapenem resistance of the NVT2001S strain. The loss of OmpK35/36 conferred 31-, 8- and 4-fold increases in the MICs of ertapenem, meropenem and doripenem, respectively, and led to carbapenem resistance. In the case of the KPC-2 and NDM-1 strains, the loss of OmpK35/36 conferred a ≤4-fold increase in the carbapenem MIC, while the KPC-2 and NDM-1 strains exhibited resistance to all four carbapenems (Table 6). Without porin loss, the CTX-M-15 and DHA-1-AmpR strains only showed a significant (≥4-fold) increase in the carbapenem MIC, while the KPC-2 and NDM-1 strains exhibited resistance to all four carbapenems (Table 6). With the loss of OmpK36, the CTX-M-15 and DHA-1-AmpR strains became resistant to both ertapenem, meropenem and doripenem. With the loss of OmpK35, the CTX-M-15, SHV-12 and DHA-1-AmpR strains became resistant to all four carbapenems, except imipenem and doripenem for the strain harboring the recombinant plasmid with *bla*<sub>ΔompK35/36</sub> (Table 6).

**Discussion**

Of the four carbapenems in this study, the loss of OmpK35/36 alone could confer carbapenem resistance, and the expression of *bla*<sub>ΔompK35/36</sub> alone was only sufficient to significantly (≥4-fold) increase the carbapenem MIC. Previous studies have shown that CTX-M β-lactamase activity against ertapenem is very low. CTX-M likely contributes to the decreased carbapenem susceptibility by binding with a high
affinity to this molecule [15,16]. Conversely, the production of KPC-2 or NDM-1 alone could render <i>K. pneumoniae</i> strains resistant to all four carbapenems, and the ertapenem MIC was the highest. Our results suggest that among the four carbapenems, ertapenem is the least active against the loss of porins, cephalosporinases and carbapenemases.

Previous studies have found that carbapenem resistance in clinical isolates can be conferred by porin loss combined with the expression of ESBLs or AmpC β-lactamases. In particular, ertapenem resistance can be caused by porin loss with the CTX-M variants [17,18]. Our results further revealed that resistance to all four carbapenems could be rendered by the loss of OmpK35/36 combined with the expression of <i>bla</i><sub>DHA-1</sub>-<i>AmpR</i> in <i>K. pneumoniae</i>. Because carbapenems are frequently utilized as drugs of last resort for the treatment of a variety of infections caused by multidrug-resistant bacteria, this finding is notable when using carbapenems for treating infections due to ESBL- or AmpC β-lactamase-producing <i>Enterobacteriaceae</i> with the loss of porins.

DHA-1 is a plasmid-encoded AmpC β-lactamase and <i>bla</i><sub>DHA-1</sub>-<i>AmpR</i> expression is transcriptionally regulated by the divergently read <i>ampR</i> gene [19]. The mechanism of AmpC induction is intimately linked to a cell wall recycling system [20-22]. AmpR can both activate and repress <i>ampC</i> expression according to its interaction with specific murein degradation products, and the murein synthesis was interfered with β-lactams [3,21]. Previous study showed that AmpR represses the synthesis of AmpC β-lactamase by 2.5-fold in the absence of an inducer, while its expression is induced more than 10-fold in the presence of a β-lactam [23]. The β-lactams also differ in their inducing abilities [3]. Of the strains harboring recombinant plasmids in this study, the DHA-1-AmpR strains showed higher MICs for many antibiotics tested compared with those of the DHA-1 strains. Whether this result is due to the expression of <i>bla</i><sub>DHA-1</sub>-<i>AmpR</i> in the presence of these antibiotics requires further studies. The DHA-1-AmpR strains with the loss of OmpK35/36 became highly resistant to multiple drugs, including the four carbapenems. However, ceftazidime should be an effective β-lactam against these strains.

Carbapenemase can effectively inactivate most β-lactam antibiotics, including carbapenems, and most carbapenemase genes are transferable. The rapid identification of carbapenemase producers is needed to prevent the development of outbreaks. Ertapenem is a sensitive indicator of AmpC induction, while its expression is induced more than 10-fold in the presence of a β-lactam [23]. Of the strains harboring recombinant plasmids in this study, the DHA-1-AmpR strains showed higher MICs for many antibiotics tested compared with those of the DHA-1 strains. Whether this result is due to the expression of <i>bla</i><sub>DHA-1</sub>-<i>AmpR</i> in the presence of these antibiotics requires further studies. The DHA-1-AmpR strains with the loss of OmpK35/36 became highly resistant to multiple drugs, including the four carbapenems. However, ceftazidime should be an effective β-lactam against these strains.

### Table 4. MICs of antibiotics against <i>K. pneumoniae</i> NVT2001S and its porin-loss mutants with AmpC β-lactamase and/or its regulator.

| Antibiotic | Minimal inhibitory concentration (μg/ml)<sup>a</sup> | Activity of Four Carbapenems
|---|---|---|
| **NVT2001S** | DHA-1-AmpR<sup>b</sup> | DHA-1 | AmpR | DHA-1-AmpR | DHA-1 | AmpR | DHA-1-AmpR | DHA-1 | AmpR | DHA-1-AmpR | DHA-1 | AmpR |
| | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> | MIC<sub>β</sub> |
| Aztreonam | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 |
| Ampicillin | &ge;16 | &ge;16 | &ge;16 | &ge;16 | &ge;16 | &ge;16 | &ge;16 | &ge;16 | &ge;16 | &ge;16 | &ge;16 | &ge;16 |
| Piperacillin/TZB | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 |
| Cefazolin | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 |
| Cephalothin | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 |
| Cefoxitin | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 | &ge;128 |
| Ceftriaxone | &le;8 | &le;8 | &le;8 | &le;8 | &le;8 | &le;8 | &le;8 | &le;8 | &le;8 | &le;8 | &le;8 | &le;8 |
| Cefpodoxime | &ge;64 | &ge;64 | &ge;64 | &ge;64 | &ge;64 | &ge;64 | &ge;64 | &ge;64 | &ge;64 | &ge;64 | &ge;64 | &ge;64 |
| Cefotaxime | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 | &ge;32 |
| Cefotaxime/CLA | 64 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| Cefazidime | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 |
| Cefazidime/CLA | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 | &ge;256 |
| Cefepime | &le;4 | &le;4 | &le;4 | &le;4 | &le;4 | &le;4 | &le;4 | &le;4 | &le;4 | &le;4 | &le;4 | &le;4 |
| Ciprofloxacin | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 | &le;1 |
| Gentamicin | &le;16 | &le;16 | &le;16 | &le;16 | &le;16 | &le;16 | &le;16 | &le;16 | &le;16 | &le;16 | &le;16 | &le;16 |

<sup>a</sup>orficase numbers indicate a significant (≥4-fold) difference in the MICs of <i>K. pneumoniae</i> NVT2001S and its derived strains, while the underlying numbers were above the breakpoint of susceptibility established by CLSI in 2013 [14].

<sup>b</sup>The β-lactamase and/or its regulator on the plasmid were shown, and the plasmid was transferred into <i>K. pneumoniae</i> NVT2001S and its porin-loss mutants.

<sup>c</sup>MIC, minimal inhibitory concentration; CLP, low-copy-number plasmid.

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Table 5. MICs of antibiotics against *K. pneumoniae* NVT2001S and its porin-loss mutants with carbapenemase.

| Antibiotic† | Minimal inhibitory concentration (µg/ml)‡ | NT | KPC-2 | ΔompK36 | ΔompK36/NDM-1 |
|--------------|------------------------------------------|-----|-------|---------|---------------|
| Aztreonam    | ≥256 ≥256 ≤1 ≤1 ≥256 ≥256 ≤1 ≤1 ≥256 ≥256 ≤1 ≤1 ≥256 ≥256 ≤1 ≤1 | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP |
| Amoxicillin  | ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 ≥32 | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP |
| Cefpodoxime  | ≤2 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 ≤16 | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP |
| Cefpodoxime/CLA | 0.5 8 64 ≥128 1 8 ≥128 16 64 ≥128 16 64 ≥128 16 64 ≥128 16 64 | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP |
| Cefpodoxime/CLA | 2 32 ≥256 ≥256 4 64 ≥256 ≥256 8 64 ≥256 ≥256 8 64 ≥256 ≥256 8 64 | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP |
| Gentamicin   | ≤4 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 ≤32 | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP | pKP | LCP | pND | LCP |

‡TZB, tazobactam with a fixed concentration of 4 µg/ml; CLA, clavulanic acid with a fixed concentration of 4 µg/ml.

The β-lactamase on the plasmid was shown, and the plasmid was transferred into *K. pneumoniae* NVT2001S and its porin-loss mutants.

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**β-lactamase-producing strains are endemic.**
Table 6. MICs of carbapenems against *K. pneumoniae* NVT2001S and its derived strains.

| Strain and carbapenem | Minimal inhibitory concentration (μg/ml) |
|-----------------------|-----------------------------------------|
|                       | None | CTX-M-15 | SHV-12 | DHA-1-AmpR | DHA-1 | AmpR | KPC-2 | NDM-1 |
| NVT2001S              |      |          |        |            |       |      |       |       |
| Ertapenem             | 0.032| 0.047    | 0.19   | 0.38       | 0.125 | 0.047| 0.75   | 0.38  | 0.047 | 0.012|
| Imipenem              | 0.25 | 0.25     | 0.25   | 0.25       | 0.38  | 0.38 | 0.25   | 0.25  | 0.25  | 0.25  |
| Meropenem             | 0.047| 0.047    | 0.064  | 0.125      | 0.064 | 0.047| 0.064  | 0.064 | 0.047 | 0.032|
| Doripenem             | 0.047| 0.047    | 0.064  | 0.094      | 0.047 | 0.047| 0.064  | 0.064 | 0.047 | 0.047|
| ΔompK35               |      |          |        |            |       |      |       |       |
| Ertapenem             | 0.047| 0.047    | 0.5    | 0.5        | 0.25  | 0.094| 1.0    | 0.75  | 0.125 | 0.023|
| Imipenem              | 0.25 | 0.25     | 0.25   | 0.25       | 0.38  | 0.38 | 0.25   | 0.25  | 0.25  | 0.25  |
| Meropenem             | 0.064| 0.047    | 0.094  | 0.125      | 0.094 | 0.094| 0.094  | 0.094 | 0.047 | 0.047|
| Doripenem             | 0.047| 0.047    | 0.064  | 0.125      | 0.064 | 0.047| 0.094  | 0.094 | 0.047 | 0.047|
| ΔompK36               |      |          |        |            |       |      |       |       |
| Ertapenem             | 0.047| 0.047    | 1.0    | 1.0        | 0.38  | 0.125| 4      | 2     | 0.19  | 0.023|
| Imipenem              | 0.25 | 0.25     | 0.38   | 0.5        | 0.38  | 1.0  | 0.5    | 0.5   | 0.25  | 0.25  |
| Meropenem             | 0.064| 0.064    | 0.19   | 0.25       | 0.19  | 0.094| 0.5    | 0.38  | 0.064 | 0.047|
| Doripenem             | 0.047| 0.047    | 0.125  | 0.25       | 0.125 | 0.094| 0.5    | 0.38  | 0.125 | 0.047|
| ΔompK35/36            |      |          |        |            |       |      |       |       |
| Ertapenem             | 1.0  | 1.5      | >32    | >32        | >32   | >32  | >32    | >32   |
| Imipenem              | 0.5  | 0.5      | 2      | 3          | 2     | 0.75 | 32     | 12    | 1.0   | 0.38 |
| Meropenem             | 0.38 | 0.5      | 6      | 8          | 4     | 1.5  | 24     | 8     | 0.75  | 0.38 |
| Doripenem             | 0.19 | 0.25     | 3      | 6          | 2     | 1.0  | 16     | 8     | 0.38  | 0.125|

*Boldface numbers indicate a significant (≥4-fold) difference in the MICs of *K. pneumoniae* NVT2001S and its derived strains, while the underlined numbers were above the breakpoint of susceptibility established by CLSI in 2013 [14].

bThe β-lactamase and/or its regulator on the plasmid were shown, and the plasmid was transferred into *K. pneumoniae* NVT2001S and its porin-loss mutants.

No, no supplemental plasmid; pCT, pSH, pDH, pKP and pND, plasmids from clinical isolates; LCP, low-copy-number plasmid.

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Supporting Information

Table S1. Oligonucleotide primers used in this study. (DOC)

Author Contributions

Conceived and designed the experiments: YKT CHL CPF JCL LKS. Performed the experiments: YKT CHL CPF JCL LKS.

References

1. Livermore DM (2012) Current epidemiology and growing resistance of gram-negative pathogens. Korean J Intern Med 27: 128-142. doi: 10.3346/kjims.2012.27.2.128. PubMed: 22707882.

2. Chong Y, Ito Y, Kamimura T (2011) Genetic evolution and clinical impact in extended-spectrum β-lactamase-producing Escherichia coli and Klebsiella pneumoniae. Infect Genet Evol 11: 1499-1504. doi: 10.1016/j.meegid.2011.06.001. PubMed: 21680785.

3. Jacoby GA (2000) AmpC β-lactamases. Clin Microbiol Rev 22: 161-182. doi:10.1128/CMR.00036-08. PubMed: 19136439.

4. Yan JJ, Hsuéh PR, Lu JJ, Chang FY, Shyr JM et al. (2006) Extended-spectrum β-lactamases and plasmid-mediated AmpC enzymes among clinical isolates of Escherichia coli and Klebsiella pneumoniae from seven medical centers in Taiwan. Antimicrob Agents Chemother 50: 1861-1864. doi:10.1128/AAC.50.5.1861-1864.2006. PubMed: 16641462.

5. Shin SY, Bae IK, Kim J, Jeong SH, Yong D et al. (2012) Resistance to carbapenems in sequence type 11 Klebsiella pneumoniae is related to DHA-1 and loss of OmpK35 and/or OmpK36. J Med Microbiol 61: 239-245. doi:10.1099/jmm.0.037036-0. PubMed: 21940650.

6. Chudácková E, Bergerová T, Fajfrlík K, Cervená D, Urbásková P et al. (2010) Carbapenem-nonsusceptible strains of Klebsiella pneumoniae. J Med Microbiol 58: 128-142. doi:10.1099/jmm.0.008094-0. PubMed: 1918225.

7. Wang XD, Cai JC, Zhou HW, Zhang R, Chen GX (2009) Reduced susceptibility to carbapenems in Klebsiella pneumoniae clinical isolates associated with plasmid-mediated β-lactamase production and OmpK36 porin deficiency. J Med Microbiol 58: 1196-1202. doi:10.1099/jmm.0.008094-0. PubMed: 19528170.

8. Nordmann P, Naas T, Poirel L (2011) Global spread of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis 17: 1791-1798. doi:10.3201/eid1710.110655. PubMed: 22000347.

9. Tsai YK, Fung CP, Lin JC, Chen JH, Chang FY et al. (2011) Klebsiella pneumoniae outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. Antimicrob Agents Chemother 55: 1485-1493. doi:10.1128/AAC.01275-10. PubMed: 21826452.

10. Ho PL, Shek RH, Chow KH, Duan RS, Mak GC et al. (2005) Detection and characterization of extended-spectrum β-lactamases among bloodstream isolates of Enterobacter spp. in Hong Kong, 2000-2002. J Antimicrob Chemother 55: 326-332.

11. SiuLK, Lin JC, Gomez E, Eng R, Chiang T (2012) Virulence and plasmid transferability of KPC Klebsiella pneumoniae at the Veterans Affairs Healthcare System of New Jersey. Microb Drug Resist 18: 380-384. doi:10.1089/mdr.2011.0241. PubMed: 22533374.

12. Ohtomo R, Saito M (2003) A new selective medium for detection of Klebsiella from dairy environments. Microbes Environ 18: 138-144. doi: 10.1264/jseme2.18.138.

13. CLSI (2012) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard–ninth edition. CLSI Document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

14. CLSI (2013). performance standards for antimicrobial susceptibility testing; Twenty-third informational supplement. CLSI Document M100-S23. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

15. Girlich D, Poirel L, Nordmann P (2009) CTX-M expression and selection of etaramep resistance in Klebsiella pneumoniae and Escherichia coli. Antimicrob Agents Chemother 53: 832-834. doi: 10.1128/AAC.01007-08. PubMed: 19029330.

16. Girlich D, Poirel L, Nordmann P (2008) Do CTX-M β-lactamases hydrolyse ertapenem? J Antimicrob Chemother 62: 1155-1156. doi: 10.1093/jac/dkn317. PubMed: 18662942.

17. Elliott E, Brink AJ, van Greune J, Els Z, Woodford N et al. (2006) In vivo development of ertapenem resistance in a patient with pneumonia caused by Klebsiella pneumoniae with an extended-spectrum β-lactamase. Clin Infect Dis 42: e95-e98. doi:10.1086/498517. PubMed: 16652304.

18. Doumith M, Ellington MJ, Livermore DM, Woodford N (2009) Molecular mechanisms disrupting porin expression in ertapenem-resistant Klebsiella and Enterobacter spp. clinical isolates from the UK. J Antimicrob Chemother 63: 659-667. doi:10.1093/jac/dkp029. PubMed: 19233898.

19. Barnaud G, Arlet G, Verdet C, Gaillot O, Lagrange PH et al. (1998) Salmonella enteritidis: AmpC plasmid-mediated inducible β-lactamase (DHA-1) with an ampR gene from Morganella morganii. Antimicrob Agents Chemother 42: 2352-2358. PubMed: 9736562.

20. Hansøn ND, Sanders CC (1999) Regulation of inducible AmpC β-lactamase expression among Enterobacteriaceae. Curr Pharm Des 5: 881-894. PubMed: 10539994.

21. Jacobs C, Frère JM, Normark S (1997) Cytosolic intermediates for cell wall biosynthesis and degradation control inducible β-lactam resistance in gram-negative bacteria. Cell 88: 823-832. doi:10.1016/S0092-8674(00)81928-5. PubMed: 9118225.

22. Jacobs C, Huang LJ, Bartowsky E, Normark S, Park JT (1994) Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J 13: 4684-4694. PubMed: 7925310.

23. Lindberg F, Westman L, Normark S (1985) Regulatory components in Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J 13: 4684-4694. PubMed: 7925310.

24. Vading M, Samuelsen O, Haldorsen B, Sundsfjord AS, Giske CG (2011) Comparison of disk diffusion, Etest and VITEK2 for detection of carbapenemase-producing Klebsiella pneumoniae with the EUCAST and CLSI breakpoint systems. Clin Microbiol Infect 17: 668-674. doi: 10.1111/j.1469-0691.2010.03299.x. PubMed: 20649801.

25. Anderson LF, Lonsway DR, Rasheed JK, Biddle J, Jensen B et al. (2007) Evaluation of methods to identify the Klebsiella pneumoniae carbapenemase in Enterobacteriaceae. J Clin Microbiol 45: 2723-2725. doi:10.1128/JCM.00015-07. PubMed: 17581947.

26. Woodford N, Dallow JW, Hill RL, Palepou MF, Pike R et al. (2007) Ertapenem resistance among Klebsiella and Enterobacter submitted in the UK to a reference laboratory. Int J Antimicrob Agents 29: 365-367. doi:10.1016/S0924-8579(07)71461-9. PubMed: 17293088.

27. Wu HS, Chen TL, Chen IC, Huang MS, Wang FD et al. (2010) First identification of a patient colonized with Klebsiella pneumoniae carrying blaBla<sup>TEM</sup> in Taiwan. J Chin Med Assoc 73: 596-598. doi:10.1016/S1726-4901(10)70129-5. PubMed: 21093828.

28. Yi H, Cho YJ, Yong D, Chun J (2012) Genome sequence of Escherichia coli J53, a reference strain for genetic studies. J Bacteriol 194: 3742-3743. doi:10.1128/JB.00641-12. PubMed: 22740669.

Activity of Four Carbapenems

Analyzed the data: YKT JCL LKS. Contributed reagents/materials/analysis tools: YKT CHL CPF JCL LKS. Wrote the manuscript: YKT CHL CPF JCL LKS. Study design: YKT CHL CPF JCL LKS Perform the experiment: YKT CHL.