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In vitro activities of carbapenems in combination with amikacin, colistin, or fosfomycin against carbapenem-resistant *Acinetobacter baumannii* clinical isolates

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ABSTRACT

Carbapenem-resistant *Acinetobacter baumannii* clinical isolates (n=23) were investigated for carbapenem resistance mechanisms and *in vitro* activities of carbapenems in combination with amikacin, colistin, or fosfomycin. Major carbapenem resistance mechanism was OXA-23 production. The vast majority of these isolates were OXA-23–producing *A. baumannii* ST195 and ST542, followed by novel STs, ST1417, and ST1423. The interuption of *carO* by a novel insertion sequence, IS*Aba40*, was found in two isolates. The combinations of imipenem and fosfomycin, meropenem and amikacin, imipenem and amikacin, and imipenem and colistin were synergistic against carbapenem-resistant *A. baumannii* by 65.2%, 46.2%, 30.8%, and 17.4%, respectively. Surprisingly, the combination of imipenem and fosfomycin was the most effective in this study against *A. baumannii*, which is intrinsically resistant to fosfomycin. Imipenem and fosfomycin inhibit cell wall synthesis; therefore, fosfomycin may be an adjuvant and enhance the inhibition of cell wall synthesis of carbapenem-resistant *A. baumannii* when combined with imipenem.

1. Introduction

Carbapenem-resistant *Acinetobacter baumannii* has emerged as a major opportunistic pathogen associated with nosocomial infection worldwide (Higgins et al., 2010; Peleg et al., 2008). The carbapenem resistance in *A. baumannii* is mediated by carbapenemase production (such as OXA-51, OXA-23, OXA-58, OXA-24, OXA-143, OXA-235, NDM, IMP, and VIM carbapenemases), reduced outer membrane proteins (OMPs) (CarO, 33–36-kDa OMP, and 43-kDa OMP), and overexpression of efflux pumps (AdeABC pump) (Higgins et al., 2013; Poirel and Nordmann, 2006). The antibiotic combination therapy may play an important role in the treatment of carbapenem-resistant *A. baumannii* infection (Viehman et al., 2014). Amikacin is an aminoglycoside antibiotic that inhibits bacterial protein synthesis by binding to 16S rRNA. The combination of β-lactams and aminoglycosides including amikacin showed synergistic effect against *A. baumannii* (Joly-Guillou et al., 1995). Colistin is the most common antibiotic used for monotherapy and combination therapy for the treatment of carbapenem-resistant *A. baumannii* infection (Viehman et al., 2014). As heteroresistance and resistance have emerged during monotherapy, colistin combination therapy is used to treat multidrug-resistant *A. baumannii* infection (Li et al., 2006; Viehman et al., 2014). Fosfomycin inhibits bacteria cell wall synthesis. Although *A. baumannii* is intrinsically resistant to fosfomycin (Lu et al., 2011), the combinations of fosfomycin and sulbactam or colistin were reported to be effective against *A. baumannii* (Santimaleeworagun et al., 2011; Sirijatuphat and Thamlikitkul, 2014). In this study, we aimed to investigate carbapenem resistance mechanisms in *A. baumannii* clinical isolates and to determine the *in vitro* effects of carbapenems in combination with amikacin, colistin, or fosfomycin.

2. Material and methods

2.1. Bacterial isolates

A total of 23 *A. baumannii* clinical isolates were collected from different patients at the King Chulalongkorn Memorial Hospital in Thailand during 2010–2011 and 2015–2016. Gram staining and conventional biochemical tests were used to identify the genus *Acinetobacter*. The $bla_{\text{OXA-51-like}}$ is an intrinsic carbapenemase gene in *A. baumannii* (Turton et al., 2006), and its presence was detected by polymerase chain reaction (PCR). The multiplex PCR for *gyrB* as described by Higgins et al (Higgins et al., 2010) was used to identify the species.

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2.2. Antibiotic susceptibility testing

The MICs of imipenem (Apollo Scientific, Manchester, UK), meropenem (Sigma-Aldrich, Steinheim, Germany), amikacin (Sigma-Aldrich), colistin (Sigma-Aldrich), and fosfomycin (Wako Pure Chemical Industries, Osaka, Japan) were determined by broth microdilution method. Fosfomycin susceptibility testing was performed by agar dilution as recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2016). The Mueller–Hinton agar (Difco, MI, USA) or cation-adjusted Mueller–Hinton broth (CAMHB) (Difco) was supplemented with 25 mg/L of glucose-6-phosphate (Sigma-Aldrich). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference control isolates for susceptibility testing. The susceptibilities of imipenem, meropenem, amikacin, and colistin were interpreted according to the CLSI guidelines (CLSI, 2016). The interpretation criteria for Enterobacteriaceae by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2016) was used for fosfomycin susceptibility.

2.3. Clonal studies

The clonal relateness of *A. baumannii* isolates was determined by multilocus sequence typing (MLST) as described by Bartual et al (Bartual et al., 2005). Briefly, genomic DNA of *A. baumannii* was extracted by PureLink Genomic DNA Mini Kit (Invitrogen, CA, USA). Seven housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*) were amplified by PCR using Master Cycler gradient instrument (Eppendrof, Hamburg, Germany) and sequenced by using the BigDye Terminator V3.1 cycle sequencing kit from the 1st Base DNA sequencing service, Malaysia. The MLST profiles were analyzed by using MLST Oxford scheme (http://pubmlst.org/abaumannii/).

2.4. Detection of carbapenemase genes

The carbapenemase genes were detected by using three multiplex PCR. Serine carbapenemase gene multiplex PCR was used to detect $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-143-like}}$, and $bla_{\text{OXA-235-like}}$ (Higgins et al., 2013). Metallocarbapenemase gene multiplex PCR was used to detect $bla_{\text{IMP-like}}$, $bla_{\text{VIM-like}}$, $bla_{\text{GIM-like}}$, $bla_{\text{SIM-like}}$, and $bla_{\text{SPM-like}}$ (Ellington et al., 2007). The third multiplex PCR was performed to detect $bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$, and $bla_{\text{KPC-like}}$.(Poirel et al., 2011).

2.5. OMP profile study

OMPs of *A. baumannii* were extracted as previously described with a slight modification (Zander et al., 2013). Briefly, mid-log phase of *A. baumannii* isolates was collected and broken by sonication (Sonics and Materials, Inc., CT, USA). The membrane fractions were collected by ultracentrifugation at 100,000*g* for 1 h at 4 °C (Beckman-Coulter, CA, USA). Then, OMPs were extracted by using 2% sodium *N*-lauryl sarconate (Merck Millipore, NJ, USA), collected by ultracentrifugation, and resuspended with phosphate buffer saline. The protein concentration was evaluated by using Bio-Rad protein assay (Bio-Rad, CA, USA). The OMPs profile were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The polyacrylamide gels were stained with Coomassie brilliant blue. The outer membrane protein (CarO, 33–36-kDa OMP, and 43-kDa OMP) expression of carbapenem-resistant isolates was compared to that of *A. baumannii* ATCC 19606, and its relative intensities were calculated by using ImageJ. The OMP genes (*carO*, 33–36-kDa OMP gene, and 43-kDa OMP gene) of isolates with reduced or loss of OMPs were amplified and sequenced by using primers listed in Table 1.

2.6. Checkerboard assay

The synergistic activities of carbapenems (imipenem and meropenem) plus amikacin, colistin, or fosfomycin were screened by checkerboard technique, which was performed in 96-well microtiter plates. Briefly, the rows of the plates contained CAMHB supplemented with two-fold serial dilution of the first antibiotic in each well, and two-fold serial dilution of the second antibiotic was added in the column of the plates. The plates were inoculated with *A. baumannii* and incubated at 35 °C for 18–24 h. Fractional inhibitory concentration index (FICI) was calculated by the summation of $\text{MIC}_{\text{drugA in combination}}/\text{MIC}_{\text{drugA alone}}$ and $\text{MIC}_{\text{drugB in combination}}/\text{MIC}_{\text{drugB alone}}$. The interpretation of FICI was as follows: ≤0.5 = synergy, >4 = antagonism, and >0.5–4 = no interaction.

2.7. Time-kill assay

The antibiotic combination which showed the highest synergistic activity (imipenem plus fosfomycin) by checkerboard technique was confirmed by time-kill study. The flasks containing CAMHB supplemented with 1× MIC and/or 0.5× MIC of each antibiotic or in combinations (supplemented with 25 mg/L of glucose-6-phosphate for fosfomycin activity) were incubated with 10^6 CFU/mL *A. baumannii* isolates in the shaking incubator at 35 °C, 120 rpm. Viable cells were quantified after 0, 2, 4, 6, 12, and 24 h of incubation by plate counting as described previously (Treyaprasert et al., 2007). The time-kill assay was done in triplicate. The synergistic activity was defined as having $2\log_{10}$ (CFU/mL)-fold reduction when compared to the most active single antibiotic. The bactericidal activity was defined as $\geq 3\log_{10}$ (CFU/mL)-fold reduction when compared to the number of viable cell at initial time point.

3. Results

3.1. Antibiotic susceptibility testing

The MICs of 6 antibiotics against 23 *A. baumannii* isolates are shown in Table 2. All of the *A. baumannii* isolates were resistant to imipenem and meropenem (MIC ≥8 mg/L) but were susceptible to colistin. According to fosfomycin MIC breakpoint interpretation for Enterobacteriaceae by EUCAST, all were resistant to fosfomycin (MIC ≥64 mg/L). Of the 23 *A. baumannii* isolates, 12 (52%) and 4 (17%) were resistant and intermediate resistant to amikacin, respectively (Table 2).

Table 1
Oligonucleotide sequences of primers used in this study.

Gene	Primer sequences (5'–3')	Length of amplicon (bp)	reference
33–36-kDa *omp*	F-AAGGTGAGGCATACGTTCCA R-TTTACGTTACCACCCCAAGC	502	(Zander et al., 2013)
43-kDa *omp*	F-ATGCTAAAAGCACAAAAACTTAC R-TTAGAATAATTTCACAGGAATATC	1327	This study
carO	F-GATGAAGCTGTTGTTCAT R-TTACCAGAAGAAGTTCAC	678	This study
Entire *carO*	F-TCAACACCACATGGATTGCT R-TTCAACAGCTTGGCGAATTT	1328	This study

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