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

Uthaibhorn Singkham-in [a], Tanittha Chatsuwan [b,*]

[a] Interdisciplinary Program of Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok, Thailand
[b] Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

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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.

* Corresponding author. Tel.: +66-2-256-4132; fax: +66-2-252-5952.
E-mail address: Tanittha.C@chula.ac.th (T. Chatsuwan).

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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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Table 2
Minimum inhibitory concentrations of antibiotics and carbapenem resistance mechanisms of *A. baumannii*.

Strain	ST type	MICs (mg/L) of antibiotics					Carbapenemase	International clone	Relative intensity of OMPs[a]		
		IPM	MEM	CT	AK	FOF			CarO	33–36-kDa OMP	43-kDa OMP
AB2	195	64(R)	64(R)	2(S)	>256(R)	128(R)	OXA-51, OXA-23	IC2	1.35	0.43	0.73
AB5	195	64(R)	128(R)	1(S))	>256(R)	128(R)	OXA-51, OXA-23	IC2	3.20	1.19	0.93
AB6	195	32(R)	32(R)	1(S)	>256(R)	128(R)	OXA-51, OXA-23	IC2	3.72	2.08	1.28
A7	195	64(R)	64(R)	1(S)	>256(R)	256(R)	OXA-51, OXA-23	IC2	3.44	2.29	1.95
AB29	542	32(R)	64(R)	1(S)	256(R)	128(R)	OXA-51, OXA-23		1.37	0.46	0.57
AB58	542	32(R)	64(R)	1(S)	32(I)	256(R)	OXA-51, OXA-23		0.14	0.29	0.51
AB97	542	32(R)	64(R)	1(S)	16(S)	128(R)	OXA-51, OXA-23		0.30	0.51	1.04
AB13	542	32(R)	64(R)	1(S)	64(R)	128(R)	OXA-51, OXA-23		1.33	0.61	0.95
AB35	1417	32(R)	64(R)	1(S)	8(S)	256(R)	OXA-51, OXA-23		2.14	1.15	1.66
A4	1417	64(R)	64(R)	1(S)	>256(R)	256(R)	OXA-51, OXA-23		2.24	1.24	1.70
AB4	1423	64(R)	64(R)	1(S)	>256(R)	256(R)	OXA-51, OXA-23		2.03	0.75	0.83
AB354	1423	64(R)	128(R)	1(S)	>256(R)	256(R)	OXA-51, OXA-23		2.05	0.87	1.18
AB1	806	32(R)	32(R)	2(S)	>256(R)	128(R)	OXA-51, OXA-23		0.56	1.23	1.15
AB3	1415	16(R)	32(R)	2(S)	0.5(S)	128(R)	OXA-51, OXA-23		1.78	0.75	0.95
AB9	514	16(R)	32(R)	2(S)	0.5(S)	128(R)	OXA-51, OXA-23		1.61	0.61	0.75
AB55	1166	32(R)	64(R)	1(S)	>256(R)	256(R)	OXA-51, OXA-23		1.80	1.28	1.75
A5	229	32(R)	64(R)	1(S)	32(I)	256(R)	OXA-51, OXA-23	IC7	1.66	1.01	1.64
A8	1418	64(R)	128(R)	1(S)	1(S)	256(R)	OXA-51, OXA-23		1.54	0.93	2.38
A9	551	64(R)	128(R)	1(S)	>256(R)	256(R)	OXA-51, OXA-23		1.05	1.45	3.22
AB227	208	16(R)	8(R)	1(S)	32(I)	128(R)	OXA-51, OXA-58	IC2	0.90	0.33	0.94
A6	1001	64(R)	64(R)	1(S)	32(I)	256(R)	OXA-51, OXA-58		1.00	1.00	0.79
AB250	1416	16(R)	16(R)	1(S)	4(S)	128(R)	OXA-51, OXA-24		0.18	0.32	0.43
A10	1426	128(R)	256(R)	2(S)	2(S)	256(R)	OXA-51, OXA-24		0.80	0.55	0.43

IPM = imipenem; MEM = meropenem; CT = colistin; AK = amikacin; FOF = fosfomycin; S = susceptible; I = intermediate resistant; R = resistant.
[a] Relative intensity of OMP bands was calculated and compared with *A. baumannii* ATCC 19606.

3.2. Carbapenemase genes

The intrinsic carbapenemase gene in *A. baumannii*, $bla_{\text{OXA-51-like}}$, was found in all 23 *A. baumannii* isolates. The $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-58-like}}$, and $bla_{\text{OXA-24-like}}$ were detected in 82.6%, 8.7%, and 8.7% of the isolates, respectively (Table 2). No $bla_{\text{OXA-143-like}}$, $bla_{\text{OXA-235-like}}$, metallocarbapenemase genes ($bla_{\text{IMP-like}}$, $bla_{\text{VIM-like}}$, $bla_{\text{GIM-like}}$, $bla_{\text{SIM-like}}$, $bla_{\text{SPM-like}}$, and $bla_{\text{NDM-like}}$), $bla_{\text{OXA-48-like}}$, and $bla_{\text{KPC-like}}$ were detected.

3.3. Clonal study

The sequence types and allelic numbers of all *A. baumannii* isolates are shown in the Supplementary Table 1. There were 15 distinct STs among 23 *A. baumannii* isolates (Supplementary Table 1), 6 of which were new STs. The most common STs were ST195 and ST542, followed by ST1417 and ST1423. All isolates which belonged to these STs carried the $bla_{\text{OXA-23-like}}$ gene. *A. baumannii* isolate AB3 and AB9 belonged to different STs (ST1415 and ST514, respectively), which were single-locus variants of *gpi* gene. They showed similar antibiotic susceptibility profiles, carbapenemase genes, and OMP expression level. The other seven isolates carrying $bla_{\text{OXA-23-like}}$ gene belonged to seven different STs. The $bla_{\text{OXA-58-like}}$–carrying isolates belonged to ST208 and ST1001. The $bla_{\text{OXA-24-like}}$–carrying isolates belonged to ST1416 and ST1426.

3.4. OMP profiles study

The CarO, 33–36-kDa OMP, and 43-kDa OMP were reduced (relative intensity <0.7) in four, nine, and four isolates, respectively (Table 2). Five isolates showed reduction in more than one OMP. Most of the isolates were OXA-23–producing *A. baumannii* ST195 and ST542 with reduced OMPs. The carbapenem MICs of OXA-23–producing *A. baumannii* with reduced OMPs and nonreduced OMPs were indifferent (16–64 and 16–128 mg/L, respectively). OXA-58–producing *A. baumannii* with reduced OMPs (AB227) showed lower carbapenem MICs than those of isolate (A6) with nonreduced OMPs (8–16 and 64 mg/L, respectively). *A. baumannii* carrying $bla_{\text{OXA-24-like}}$ isolate AB250 showed reduced CarO, 33–36-kDa OMP, and 43-kDa OMP but had

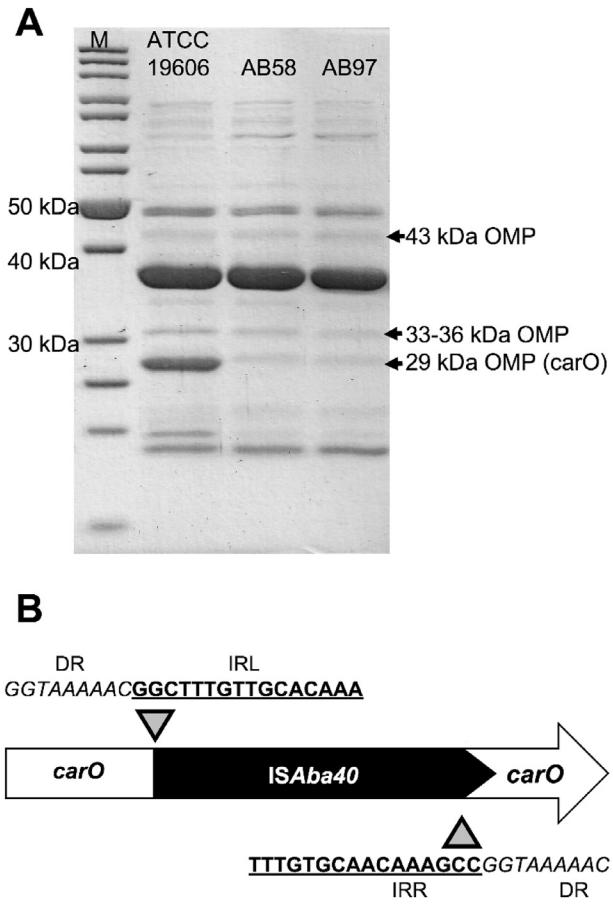

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of outer membrane protein profiles of *A. baumannii* ATCC 19606, isolate AB58, and isolate AB97 (A). The genetic scheme of interrupted *carO* by novel insertion sequence, IS*Aba40* of *A. baumannii* isolate AB58 (B).

lower carbapenem MICs (16 mg/L) than those of isolate A10 (128–256 mg/L) which showed reduced 33–36-kDa OMP and 43-kDa OMP. These results suggest that the OMP expression was not correlated with carbapenem MIC levels.

All isolates with reduced OMPs were amplified for all three OMP genes. We found unexpected results from isolate AB58 and AB97. OMP profile for both isolates and genetic scheme of *carO* of isolate AB58 are shown in Fig. 1. The PCR product from these isolates were 1700 bp, which was longer than the expected size of 678 bp. Nucleotide sequences for the entire *carO* revealed that there was a disruption in its gene due to the insertion of IS*Aba40* (https://www-is.biotoul.fr). The nucleotide sequences from isolate AB58 and AB97 were identical and have been submitted to the GenBank database under the accession numbers KX987115 and KX987116, respectively. The new insertion sequence, IS*Aba40*, was 1039 bp long (933-bp open reading frame), encoded a putative transposase, and was bound to 16-bp inverted repeat sequences with a 9-bp (5′GGTAAAAAC3′) duplication site (Fig. 1). The results suggest that the transposition of IS*Aba40* was cut and pasted into *carO* at the duplication site. IS*Aba40* belongs to the IS*5* family and encodes the transposase that contains the DDE motif. Our results showed that there was no difference between carbapenem MICs of isolates with disrupted *carO* by IS*Aba40* (AB58 and AB97) and intact *carO* (AB13 and AB29) isolates with the same ST. These data suggest that CarO disruption does not play a role in carbapenem resistance for these *A. baumannii* isolates.

3.5. Checkerboard assay

The results of 6 antibiotic combinations against 23 carbapenem-resistant *A. baumannii* by checkerboard assay are shown in Table 3. *A. baumannii* isolates with amikacin MIC >256 mg/L were excluded from the study that combined other antibiotics with amikacin. All antibiotic combinations showed synergism and no interaction against *A. baumannii* isolates, and no antagonism was observed in this study. The most effective combination was imipenem plus fosfomycin [synergism against 15/23 isolates (65.2%)], followed by meropenem plus amikacin [synergism against 6/13 isolates (46.2%)], imipenem plus amikacin [synergism against 4/13 isolates (30.8%)], and imipenem plus colistin [synergism against 4/23 isolates (17.4%)]. No synergy was observed with meropenem plus colistin or with meropenem plus fosfomycin combinations. According to the clonal study, the best combination (imipenem and fosfomycin) showed synergistic effects against *A. baumannii* belonging to various STs. No correlation between activities of antibiotic combinations and STs was found.

According to the study of carbapenem resistance mechanisms, the combination of imipenem and fosfomycin showed synergy against 11 $bla_{\text{OXA-23-like}}$–carrying *A. baumannii* isolates with reduced OMPs (5 isolates) and nonreduced OMPs (6 isolates). The synergism of this combination was found in all $bla_{\text{OXA-58}}$–carrying isolates (AB227 and A6) and $bla_{\text{OXA-24-like}}$–carrying isolates (AB250 and A10). The synergistic activities of meropenem plus amikacin and imipenem plus colistin were observed only in $bla_{\text{OXA-23-like}}$–carrying *A. baumannii* isolates, whereas the synergism of imipenem plus amikacin was found in three $bla_{\text{OXA-23-like}}$–carrying isolates and one $bla_{\text{OXA-24-like}}$–carrying isolate. These data suggest that the effects of antibiotic combinations against *A. baumannii* were not associated with carbapenem resistance mechanisms.

3.6. Time-kill assay

For time-kill studies, nine *A. baumannii* isolates with different carbapenem resistance mechanisms and belonging to different STs were exposed to combinations of imipenem and fosfomycin at concentrations of 1× or 0.5× the MICs of each drug. The combination of 1× MIC of imipenem and 1× MIC of fosfomycin showed both synergy and bactericidal activity in five isolates of different STs carrying $bla_{\text{OXA-23}}$ (AB2, AB3, AB29, AB58, and A4), one isolate (AB227) carrying $bla_{\text{OXA-58}}$, and one isolate (A10) carrying $bla_{\text{OXA-24}}$ (Fig. 2). The combination of 0.5× MIC of imipenem and 1× MIC of fosfomycin showed synergy against isolate A6 carrying $bla_{\text{OXA-58}}$ (Fig. 2G). No synergy was observed against isolate AB250 (Fig. 2H). The results suggest that the combination of imipenem and fosfomycin is effective against carbapenem-resistant *A. baumannii* isolates.

Table 3
Results of the activity of antibiotic combinations against *A. baumannii* by checkerboard assay.

Strain	ST type	FICI of antibiotic combinations (interpretation)					
		IPM + AK	IPM + CT	IPM + FOF	MEM + AK	MEM + CT	MEM + FOF
AB2	195	ND	0.63 (N)	0.31 (S)	ND	0.75 (N)	0.75 (N)
AB5	195	ND	2.00 (N)	0.56 (N)	ND	1.00 (N)	1.00 (N)
AB6	195	ND	2.00 (N)	0.50 (S)	ND	0.63 (N)	1.00 (N)
A7	195	ND	0.63 (N)	1.00 (N)	ND	0.75 (N)	2.00 (N)
AB29	542	0.31 (S)	2.00 (N)	0.31 (S)	0.38 (S)	0.75 (N)	0.53 (N)
AB58	542	0.75 (N)	2.00 (N)	0.38 (S)	0.38 (S)	0.63 (N)	2.00 (N)
AB97	542	0.50 (S)	2.00 (N)	0.31 (S)	0.50 (S)	0.63 (N)	1.00 (N)
AB13	542	0.75 (N)	0.75 (N)	0.75 (N)	0.38 (S)	1.00 (N)	0.75 (N)
AB35	1417	0.75 (N)	0.50 (S)	0.50 (S)	0.50 (S)	0.56 (N)	0.75 (N)
A4	1417	ND	0.75 (N)	0.50 (S)	ND	1.00 (N)	1.00 (N)
AB4	1423	ND	2.00 (N)	0.50 (S)	ND	1.00 (N)	1.00 (N)
AB354	1423	ND	0.50 (S)	0.53 (N)	ND	0.75 (N)	0.75 (N)
AB1	806	ND	0.63 (N)	0.56 (N)	ND	0.75 (N)	0.75 (N)
AB3	1415	0.75 (N)	0.63 (N)	0.31 (S)	0.50 (S)	2.00 (N)	0.75 (N)
AB9	514	0.75 (N)	0.63 (N)	0.31 (S)	1.00 (N)	0.75 (N)	0.53 (N)
AB55	1166	ND	0.50 (S)	1.00 (N)	ND	0.63 (N)	0.75 (N)
A5	229	0.63 (N)	0.75 (N)	2.00 (N)	0.63 (N)	1.00 (N)	1.00 (N)
A8	1418	0.50 (S)	0.75 (N)	0.75 (N)	0.75 (N)	2.00 (N)	2.00 (N)
A9	551	ND	0.50 (S)	0.50 (S)	ND	0.75 (N)	0.56 (N)
AB227	208	0.75 (N)	0.75 (N)	0.31 (S)	1.00 (N)	0.63 (N)	0.75 (N)
A6	1001	0.38 (S)	1.00 (N)	0.50 (S)	0.75 (N)	0.75 (N)	0.75 (N)
AB250	1416	0.75 (N)	2.00 (N)	0.50 (S)	1.00 (N)	0.63 (N)	1.00 (N)
A10	1426	0.75 (N)	0.75 (N)	0.50 (S)	1.00 (N)	1.00 (N)	0.75 (N)

FICI = fractional inhibitory concentration index; ND = not determined (isolates with amikacin MIC >256 mg/L were excluded); N = no interaction; S = synergy; IPM = imipenem; MEM = meropenem; CT = colistin; AK = amikacin; FOF = fosfomycin.

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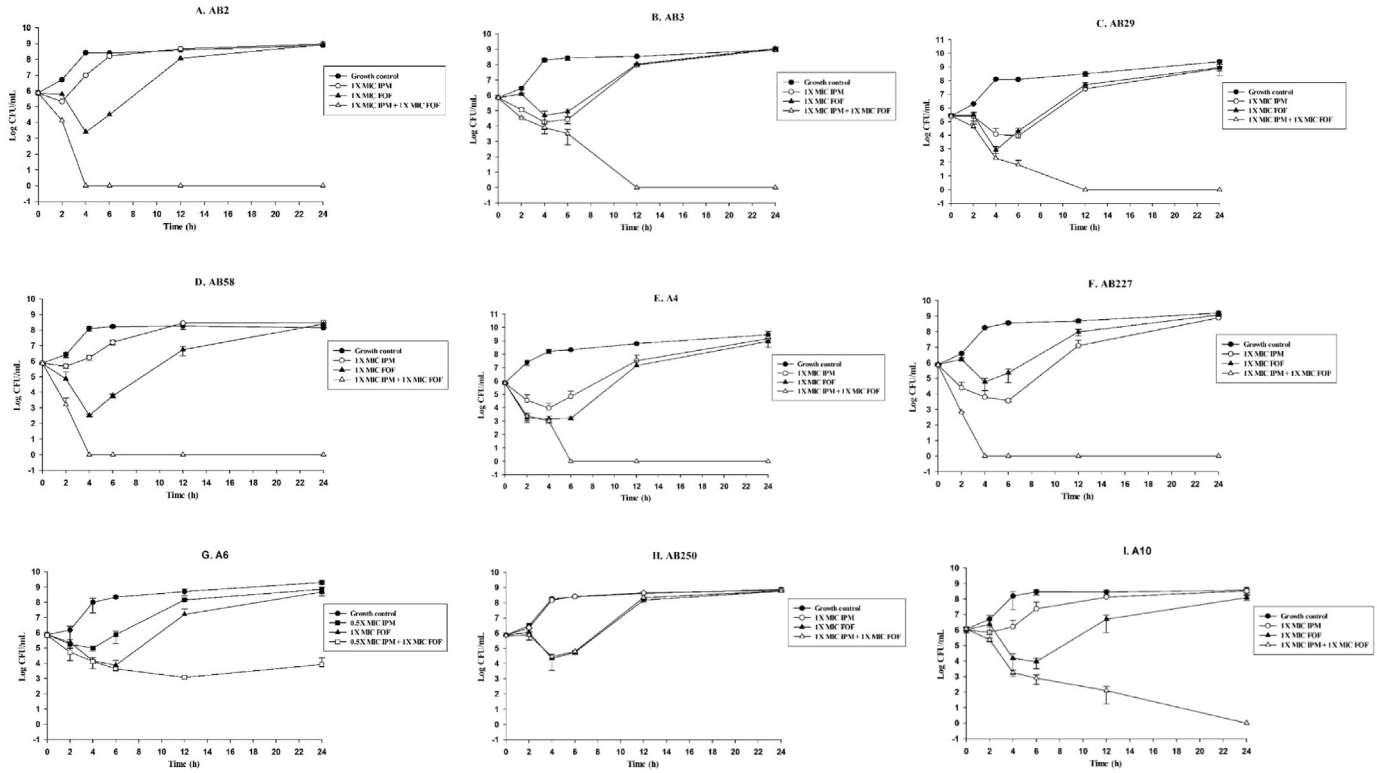

Fig. 2. Time-kill curves of imipenem in the combination with fosfomycin against nine *A. baumannii* isolates: AB2 (A), AB3 (B), AB29 (C), AB58 (D), A4 (E), AB227 (F), A6 (G), AB250 (H) and A10 (I).

4. Discussion

The emergence of antibiotic resistance in *A. baumannii* especially carbapenem resistance has been an important problem in the healthcare setting (Potron et al., 2015). OXA-23 production was the major mechanism of carbapenem resistance in *A. baumannii* isolates in this study and worldwide (Mugnier et al., 2010; Wu et al., 2015). The clonal spread (ST195 and ST542) and wide spread of OXA-23–carrying *A. baumannii* isolates were observed in this study. The ST195 *A. baumannii* isolates were reported in Thailand and Malaysia (Kim et al., 2013), while ST542 *A. pittii* was isolated from Czech Republic (Nemec et al., 2015). OXA-58– and OXA-24–producing *A. baumannii* were polyclonal. Six isolates carrying OXA-23, one isolate carrying OXA-58, and one isolate carrying OXA-24 belonged to six new STs. The results indicate that there were a variety of unique carbapenem-resistant *A. baumannii* clones. The reduced and nonreduced CarO OMPs *A. baumannii* carrying OXA-23 ST542 showed the same carbapenem MIC result. These results are similar to previous reports which showed that the carbapenem MICs were indifferent in reduced and nonreduced CarO expression *A. baumannii* carrying OXA-23 (Fonseca et al., 2013) and in reduced and nonreduced CarO and 43-kDa OMP *A. baumannii* carrying OXA-143 (Mostachio et al., 2012).

Our data showed that the best effective antibiotic combination was imipenem and fosfomycin. Surprisingly, *A. baumannii* has been reported to be intrinsically resistant to fosfomycin (Lu et al., 2011), but fosfomycin in combination with imipenem showed synergistic effect against carbapenem-resistant *A. baumannii*. The study by Martinez-Martinez et al., which *in vitro* testing of 34 *A. baumannii* isolates (of which 7 isolates were resistant to imipenem) revealed synergy between imipenem and fosfomycin in one isolate (Martinez-Martinez et al., 1996). The combination of imipenem and fosfomycin showed synergism against 46.7% of multidrug-resistant *P. aeruginosa* (Samonis et al., 2012). However, no synergism was found against carbapenem-resistant *A. baumannii* carrying OXA-143 (Leite et al., 2016). In our study, the combination of 1× MIC of imipenem (16–64 mg/L, and 128 mg/L of isolate A10) and 1× MIC of fosfomycin (128–256 mg/L) showed synergism and bactericidal effect against most *A. baumannii* isolates. Fosfomycin, when combined with imipenem, may enhance the inhibition of bacterial cell wall synthesis. According to a pharmacokinetic study, the maximum plasma fosfomycin concentration exceeded 307 mg/L after 8 g of intravenous administration (Pfausler et al., 2004). The administration of 1 g of imipenem intravenously had a plasma level of 90.1 mg/L (Novelli et al., 2005). In this study, the concentrations of imipenem and fosfomycin were synergistic and had lower drug concentration than those reported in pharmacokinetic studies (except for isolate A10). According to a pharmacokinetic/pharmacodynamic study, continuous administration of 16–24 g of fosfomycin with 3–6 g of carbapenems was considered optimal dose against non–multidrug-resistant *P. aeruginosa* (of which 40% of isolates are resistant to carbapenems) (Asuphon et al., 2016). Thus, a combination of imipenem and fosfomycin may be an alternative therapy for the treatment of carbapenem-resistant *A. baumannii*.

From the checkerboard assay, a combination of imipenem and amikacin was 30.8% effective against carbapenem-resistant *A. baumannii*, whereas a combination of imipenem and colistin was 17.4% effective. The synergy of imipenem with amikacin in our study (30.8%) was lower than that in the study of Martinez-Martinez et al. (Martinez-Martinez et al., 1996) which had a 41% synergy against 34 *A. baumannii* isolates (including all 7 imipenem-resistant isolates). Our results were different when compared to previous studies which showed that there was no synergism when the combination of these drug was used against carbapenem-resistant *A. baumannii* carrying $bla_{\text{OXA-143}}$ (Leite et al., 2016). Another study which used the same methods as our study showed no synergism of imipenem plus colistin against $bla_{\text{OXA-23}}$–carrying *A. baumannii* (Santimaleeworagun et al., 2011). Fortunately, no antagonism effect was found in this study. The limitation of this study was that there were a low number of *A. baumannii* isolates tested; thus, it was hard to find out the predicable criteria for antibiotic synergism. Even though *in vitro* synergy effects of antibiotic combination against carbapenem-resistant *A. baumannii*

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were shown in this study, *in vivo* and clinical studies are required to further investigate these activities.

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