

IN VITRO ACTIVITY OF COLISTIN OR SULBACTAM IN COMBINATION WITH FOSFOMYCIN OR IMIPENEM AGAINST CLINICAL ISOLATES OF CARBAPENEM-RESISTANT *ACINETOBACTER BAUMANNII* PRODUCING OXA-23 CARBAPENEMASES

Wichai Santimaleeworagun¹, Payom Wongpoowarak¹, Pantip Chayakul²,
Sutthiporn Pattharachayakul¹, Pimpimon Tansakul³ and Kevin W Garey⁴

¹Department of Clinical Pharmacy, ³Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, ²Department of Medicine, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand; ⁴Department of Clinical Sciences and Administration, College of Pharmacy, University of Houston, Houston, Texas, USA

Abstract. This study investigated the *in vitro* activity of colistin or sulbactam in combination with fosfomycin or imipenem against eight strains of carbapenem-resistant *A. baumannii* (CRAB). The eight CRAB clinical isolates were collected from hospitalized patients admitted to Songklanagarind Hospital in southern Thailand during January-December 2008. The isolates were divided into 4 different patterns of clonal relationships using the Repetitive Extragenic Palindromic-Polymerase Chain Reaction method (REP-PCR). The *in vitro* activity of combination antibacterial agents against these isolates were determined by checkerboard and time-kill methods. All isolates producing OXA-23 carbapenemases were universally susceptible to colistin but intermittently susceptible to other antimicrobial agents. A checkerboard assay showed the synergistic effects of sulbactam plus fosfomycin and colistin plus fosfomycin in 75% and 12.5% of isolates, respectively. Sulbactam at a concentration of 1 x MIC plus fosfomycin at 1 x MIC or at 1/4 x MIC showed synergism in 75% and 37.5% of clinical isolates, respectively. Bactericidal activity was observed for up to 12 hours of incubation. There was no synergism between colistin and sulbactam, sulbactam and imipenem, and colistin and imipenem, against the tested isolates. Combined use of sulbactam and fosfomycin may provide an alternative therapeutic option for CRAB infections.

Key words: *Acinetobacter baumannii*, carbapenem resistant, colistin, sulbactam, fosfomycin, imipenem, carbapenemase

INTRODUCTION

Acinetobacter baumannii, a gram-

Correspondence: Wichai Santimaleeworagun, Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla 90112, Thailand.
Tel: 66 (0) 7442 8222; Fax: 66 (0) 7442 8222
E-mail: wichai1234@gmail.com

negative bacillus, causes nosocomial infections worldwide. In the United States, *Acinetobacter* species were ranked the second leading cause of mortality (43.4%) in patients admitted to intensive care units (Wisplinghoff *et al*, 2004). The National Antimicrobial Resistance Surveillance Center, Thailand (NARST) found *A. baumannii* was the fourth most

common clinical pathogen isolated from specimens studied in 2006 (NARST, 2009). *A. baumannii* related infections are associated with adverse outcomes, including increased mortality, prolonged hospitalization, additional hospital costs, and reduced patient functional status (Lee *et al*, 2007; Tseng *et al*, 2007; Young *et al*, 2007). At Songklanagarind Hospital, a tertiary care hospital located in southern Thailand, Jamulitrat *et al* (2009) found mortality rates were higher in patients with carbapenem-resistant *A. baumannii* (CRAB; 52.2%) than carbapenem-sensitive *A. baumannii* bacteremia (19.9%). At present, the optimum treatment for multi-drug resistant *A. baumannii* (MDR-AB) has not been established. In previous *in vitro* studies, colistin and rifampicin, sulbactam and imipenem and rifampicin and imipenem have been shown to have a synergistic effect on MDR-AB (Song *et al*, 2007; Tripodi *et al*, 2007). However, a synergistic effect with these drug combinations has not been seen with resistant isolates.

In Thailand, colistimethate and cefoperazone/sulbactam are antimicrobial agents used for treating multi-drug resistant gram-negative bacterial (GNB) infections, in particular, infections due to *Pseudomonas aeruginosa* and *A. baumannii*. Sulbactam, a β -lactamase inhibitor, is combined with β -lactam antibiotics (eg, ampicillin, cefoperazone) for inhibiting β -lactamases, but sulbactam itself also has activity against *A. baumannii* (Corbella *et al*, 1998). The NARST survey in 2006 (NARST, 2009) found 58% of *A. baumannii* isolates were susceptible to cefoperazone/sulbactam. In contrast, at our institution during 2004-2008, 90% of *A. baumannii* isolates were sensitive to colistin and cefoperazone/sulbactam (unpublished data). Fosfomycin has been shown to have activity against gram-positive bacteria and

some GNB, including extended-spectrum β -lactamase-producers (de Cueto *et al*, 2006; Falagas *et al*, 2008a). Falagas *et al* (2008b) found the MIC of fosfomycin against MDR-AB strains ranged from 64 to >512 mg/l. In our setting, the MIC₅₀ and MIC₉₀ of fosfomycin against MDR-AB were 64 and 96 mg/l, respectively (Hortiwakul *et al*, 2009). This *in vitro* activity of fosfomycin against MDR-AB is within possible therapeutic concentrations (Bando and Toyoshima, 1984). Since the *in vitro* activity of fosfomycin in combination with other agents against MDR-AB has not previously been reported, we investigated the combination of colistin or sulbactam with fosfomycin or imipenem against *in vitro* CRAB infections to evaluate the potential synergistic or antagonistic effects of these combinations.

MATERIALS AND METHODS

Bacterial strains

All clinical isolates were obtained from inpatients admitted at Songklanagarind Hospital, a university-affiliated medical school in southern Thailand during January-December 2008. CRAB was defined as isolates resistant to imipenem. Resistance was defined as a MIC \geq 16 mg/l according to the Clinical and Laboratory Standards Institute (CLSI, 2009). The clinical isolates of CRAB were obtained from sterile sites (eg, blood, cerebrospinal fluid, bone marrow, peritoneal fluid, pleural fluid, synovial fluid). Eight CRAB specimens were collected; four isolates had a MIC against imipenem of 16 mg/l, and the others had a MIC \geq 32 mg/l. MDR-AB was defined as isolates resistant to carbapenem and at least two antibiotics from the following: piperacillin/tazobactam, ciprofloxacin, amikacin, gentamicin, cefoperazone/sulbactam or

colistin. All CRAB strains were kept at -20°C until tested.

Polymerase chain reaction-based DNA fingerprint

The REP-PCR method was used to interpret the clonal relationships of the isolates. In this study, genomic DNA was extracted and the PCR technique was used to amplify positions in the REP region. Briefly, selected strains were cultured in Mueller-Hinton broth (Difco, Detroit, MI) and incubated at 37°C for 18 hours. The genomic DNA was prepared using the DNeasy Qiagen kit (Qiagen, Valencia, CA). The purified DNA was kept at -20°C until PCR testing was done. The PCR mixture consisted of 1 µl of genomic DNA, 10 mM of each forward and reverse primer (REP-forward: 5'-IIIGC-GCCGICATCAGGC-3' and REP-reverse: 5'-ACGTCTTATCAGGCCTAC-3') (Bou *et al*, 2000), 0.2 mM of deoxy-nucleotide triphosphate and 1.5 mM MgCl₂ in 5x GoTaq[®] buffer. The volume of GoTaq[®] polymerase (Promega, Madison, WI) was 1.25 U. The total volume of the PCR mixture was 50 µl. Thermocycling was carried out with Takara thermocycler dice, starting with denaturation at 94°C for 10 minutes, followed by 30 cycles of 94°C for 1 minute, 45°C for 1 minute (annealing temperature), 72°C for 2 minutes and a final extension at 72°C for 16 minutes. The PCR products were evaluated by electrophoresis in 1.2% of agarose gel, stained with ethidium bromide (Invitrogen, Carlsbad, CA). The bands of nucleotides were detected by UV transillumination and photographed with a camera. An isolate was considered a different clone if its REP-PCR pattern differed from the others by more than three bands (Bou *et al*, 2000).

Amplification of resistant genes

The couple primers of drug resis-

tant genes, *bla*_{IMP-like'}, *bla*_{OXA-23'}, *bla*_{OXA-40'}, *bla*_{OXA-58'}, *bla*_{SIM-1'}, *bla*_{VIM-1} and *bla*_{VIM-2'} were designed from the nucleotide sequence of each gene retrieved from GenBank. The accession numbers of each gene are shown in Table 1. Amplification was carried out under the following PCR conditions, 94°C for 5 minutes, followed by 30 cycles of first 94°C for 1 minute then annealing for 2 minutes. The annealing temperatures were optimized for each primer set based on the melting temperature of the primer, followed by 72°C for 2 minutes and a final extension at 72°C for 10 minutes. The PCR products were evaluated by agarose gel electrophoresis, ligated with the Strataclone PCR cloning kit (Stratagene, La Jolla, CA) and transferred to *E. coli*. The plasmid from the overnight *E. coli* culture was purified and used for sequencing analysis.

Determination of minimum inhibitory concentration

The MICs of the antimicrobial agents were determined by the agar dilution method using Mueller-Hinton agar (Difco) plates containing dilutions of the antimicrobial agents, according to CLSI protocol (CLSI, 2009). The lowest concentration of an antimicrobial agent that inhibited visible growth of the organism was defined as the MIC. The antimicrobial agents used in this study were: fosfomycin disodium salt (Sigma Chemical, St Louis, MO), supplemented with 25 mg/l of glucose-6-phosphate (G-6-P sodium salt; Sigma Chemical), colistin sulfate (Sigma Chemical), sulbactam (DeF Pharmaceutical Chemical Service, Shanghai, China), and imipenem (Merck, Rahway, NJ). The quality controlled strains was *E. coli* ATCC 25922, [Department of Medical Sciences Type (DMST) culture collection, Bangkok, Thailand] was used to monitor the accuracy of the MIC determination. The MIC results were interpreted according

Table 1
Oligonucleotide primer design for resistance genes in *A. baumannii*.

Gene	Primer information	Size of product (bp)	Accession number
<i>bla</i> _{IMP-like}	F- 5'CTRCCGCAGSAGMGBCTTTTG3' R- 5'AACCAGTTTTGCHTTACCAT3'	587	AJ640197 AJ243491 AY590475 AF290912 EF127959 AB074436 AB184977 AJ132105
<i>bla</i> _{OXA-23}	F- 5'GGAATTCCATGAATAAATATTTTA3' R- 5'GGATCCCCTTAAATAATATTCAGC3'	822	
<i>bla</i> _{OXA-40}	F- 5'GGAATTCCATGAAAAAATTTATAC3' R- 5'GGATCCCCTTAAATGATTCCAAGA3	828	AF509241
<i>bla</i> _{OXA-58}	F- 5'GGAATTCCATGAAATTATTA AAAA3' R- 5'GGATCCCCTTATAAATAATGAAAA3'	843	EU107365
<i>bla</i> _{SIM-1}	F- 5'GGAATTCCATGAGA ACTTTTATTGA3' R- 5'GGATCCCCTTAAATTAATGAGCGGC3'	741	AY887066
<i>bla</i> _{VIM-1}	F- 5'GGAATTCCATGTTAAAAGTTATTA3' R- 5'GGATCCCCTACTCGGCGACTGAG3'	801	DQ112355
<i>bla</i> _{VIM-2}	F- 5'GGAATTCCATGTTCAA ACTTTTGA3' R- 5'GGATCCCCTACTCAACGACTGAG3'	801	AF291420

to CLSI breakpoint criteria (CLSI, 2009). Colistin and sulbactam susceptibilities were classified using Comité de l'Antibiogramme de la Société Française de Microbiologie criteria (CA-SFM, 2009) giving breakpoints for colistin and sulbactam of ≤ 2 and ≤ 8 mg/l, respectively. Due to the lack of a standard MIC breakpoint for fosfomycin against *A. baumannii*, the MIC resistance breakpoint of ≥ 32 mg/l was used for fosfomycin, the same as the breakpoint against Enterobacteriaceae and *P. aeruginosa* (CA-SFM, 2009).

Synergistic testing

The synergistic effect of antimicrobial combinations were assessed using a checkerboard technique. This method was used to select the synergistic effect

of paired antimicrobial combinations in time-kill studies. Briefly, the checkerboard technique consists of columns in which each well is filled by twofold serial dilution with the first antibiotic and rows in which each well is filled by serial dilution with the second antibiotic. This technique was performed with Mueller-Hinton broth (Difco, Detroit, MI), and samples were incubated at 37°C for 18 hours (Pillai *et al*, 2005). In this study, the synergistic effect for each of the antimicrobial combinations was determined: colistin with sulbactam, colistin with fosfomycin, colistin with imipenem, sulbactam with fosfomycin, and sulbactam with imipenem. The fractional inhibitory concentration indices (FICI) were calculated, where $FICI = MIC_A/MIC_{A/B} + MIC_B/MIC_{B/A}$. The

Table 2

Characteristics of the eight carbapenem-resistant *A. baumannii* (CRAB) isolates studied.

Strains	Ward ^a	Specimen	Antimicrobial resistance profile ^b	REP-PCR group ^c
AB23	Med	Blood	AMK, AMP, CAZ, CIP, GEN, IPM, MEM, SXT, TZP	A
AB54	NICU	Tissue	AMK, AMP, CAZ, CIP, GEN, IPM, MEM, SXT, TZP	A
AB164	PICU	Blood	AMK, AMP, CAZ, CIP, GEN, IPM, MEM, SCFP, SXT, TZP	B
AB167	MICU	Blood	AMK, AMP, CAZ, CIP, GEN, IPM, MEM, SCFP, SXT, TZP	B
AB198	NeuSur	CSF	AMP, CAZ, CIP, IPM, MEM, SCFP, TZP	D
AB307	NICU	Blood	AMK, AMP, CAZ, SCFP, CIP, GEN, IPM, MEM, SCFP, SXT, TZP	C
AB313	Sur	Ascitic fluid	AMK, AMP, CAZ, CIP, GEN, IPM, MEM, SCFP, SXT, TZP	A
AB315	SICU	Blood	AMK, AMP, CAZ, CIP, GEN, IPM, MEM, SCFP, SXT, TZP	C

^aMed, medicine; MICU, medical intensive care unit; NICU, neonatal intensive care unit; NeuSur, neurosurgery; SICU, surgical intensive care unit; Sur, surgery;

^bAMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; SCFP, cefoperazone-sulbactam; CIP, ciprofloxacin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; SXT, sulfamethoxazole-trimethoprim; TZP, piperacillin-tazobactam

^cREP-PCR group, Repetative Extragenic Palindromic-Polymerase Chain Reaction used to determine the clonal relationships of the isolates.

results of the FICI for each of the paired antibiotics were interpreted as: ≤ 0.5 = synergistic, $> 0.5 - 4.0$ = no interaction, and ≥ 4 antagonistic effect.

Time-kill studies

The chosen optimum combinations of antibiotics depended on the synergistic activity assessed using the checkerboard method, and the antibiotic concentration would be based on $1 \times$ MIC for colistin and sulbactam and $1/4 \times$ MIC for the combined drug in each synergistic drug-combination. The synergistic effects of the drug combinations against the isolated strains were evaluated, together with bactericidal activity at 0, 2, 4, 6, 12 and 24 hours of incubation following the inter-

pretation of combinations of antibiotics and bactericidal activity. Synergy was defined as a $\geq 2 \log_{10}$ decrease in cfu/ml of the combination compared with the cfu/ml of the most active mono-antimicrobial agent. Bactericidal activity was defined as a $\geq 3 \log_{10}$ decrease in cfu/ml compared with the starting colony (Pillai *et al*, 2005).

RESULTS

The eight isolates of CRAB were obtained from various sterile sites. Using REP-PCR DNA profiling, there were 4 groups of clonal relationships divided into patterns A-D (Table 2 and Fig 1). The MIC range was lowest for colistin (0.5-1 mg/l) followed by imipenem (16-32 mg/l),

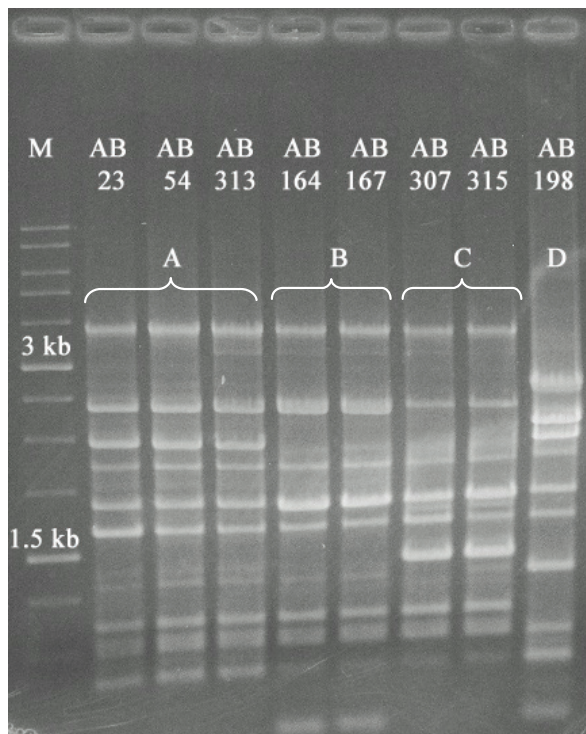


Fig 1—Pattern obtained with Repetitive Extragenic Palindromic-Polymerase Chain Reaction (REP-PCR), evaluated by electrophoresis in 1.2% agarose gel, stained with ethidium bromide. The letters above each lane indicate the strain. M, DNA molecular weight marker in kilobase unit (kb). Using the REP-PCR method, there were 4 groups divided into patterns A-D.

sulbactam (8-64 mg/l), and fosfomycin (128-512 mg/l) (Table 4). Upon evaluating for resistant genes, all isolates had the *bla*_{OXA-23} gene but were negative for all the other genes (*bla*_{OXA-40}, *bla*_{OXA-58}, *bla*_{IMP-like}, *bla*_{SIM-1}, *bla*_{VIM-1} and *bla*_{VIM-2}).

The data from the checkerboard studies are shown in Table 3. A synergistic effect was seen for colistin and fosfomycin in 1 out of 8 isolates (12.5%) and for sulbactam and fosfomycin in 6 out of 8 isolates (75%). There were no synergistic effects seen with the combinations of

colistin and sulbactam, sulbactam and imipenem, and colistin and imipenem. There were no antagonistic effects observed with any of the combinations of antimicrobial agents studied (Table 4).

With the time-kill studies, the combination of sulbactam and fosfomycin at concentrations equal to the MIC of the organism had synergistic effects in 6 of 8 isolates (75%) (Fig 2A-H). Bactericidal activity within twelve hours was observed for almost tested isolates using these concentrations. Decreasing fosfomycin to a concentration of 1/4 the MIC of the organism with sulbactam at the MIC for the organism showed synergy in 3 of 8 isolates (37.5%).

DISCUSSION

A. baumannii is an important nosocomial infection in ventilator-associated pneumonia, bacteremia, urinary tract infection, meningitis, and peritonitis (Bergogne-Bérézin and Towner, 1996). Increased antibiotic resistance by *A. baumannii* dramatically limits the potential choices of antimicrobial agents (Bergogne-Bérézin and Towner, 1996). Several studies have reported the possible mechanisms of resistance for *A. baumannii*. These include enzymatic lysis [including oxacillinases (OXA), MBL, Amp-C, aminoglycoside-modifying enzyme], loss of porins, efflux pump, or changing of penicillin binding proteins (Bergogne-Berezin and Towner, 1996; Bonomo and Szabo, 2006). This high level of *A. baumannii* resistance presents clinical challenges for appropriate treatment. Investigation of combined antimicrobial agents with activity against resistant strains of *A. baumannii* is needed.

Table 3

Determination of MICs of various antimicrobial agents against the eight clinical strains of carbapenem-resistant *A. baumannii* (CRAB) by agar dilution method.

Strains	MICs (mg/l) of various antimicrobial agents (interpretation) ^a			
	Imipenem	Colistin	Sulbactam	Fosfomycin
AB23	32 (R)	1 (S)	8 (S)	128 (R)
AB54	16 (R)	1 (S)	8 (S)	128 (R)
AB164	16 (R)	1 (S)	32 (R)	128 (R)
AB167	32 (R)	1 (S)	32 (R)	256 (R)
AB198	32 (R)	1 (S)	32 (R)	128 (R)
AB307	64 (R)	1 (S)	64 (R)	128 (R)
AB313	16 (R)	1 (S)	16 (R)	128 (R)
AB315	16 (R)	0.5 (S)	64 (R)	512 (R)

^aBreakpoint criteria were: imipenem: susceptible, ≤ 4 mg/l; intermediate, 8 mg/l; resistant, ≥ 16 mg/l according to CLSI. Colistin: susceptible, ≤ 2 mg/l; resistant, ≥ 4 mg/l according to CA-SFM. Sulbactam: susceptible, ≤ 8 mg/l according to CA-SFM. Fosfomycin: resistant, ≥ 32 mg/l according to CA-SFM.

Table 4

Testing of coupled antimicrobial agents against carbapenem-resistant *A. baumannii* (CRAB) clinical isolates by checkerboard technique.

Strains	FICI for combined antimicrobial agents ^a				
	Colistin + sulbactam	Colistin + imipenem	Colistin + fosfomycin	Sulbactam + imipenem	Sulbactam + fosfomycin
AB23	0.96 (I)	0.94 (I)	1.17 (I)	0.63 (I)	0.32 (S)
AB54	1.54 (I)	0.56 (I)	1.04 (I)	0.52 (I)	0.49 (S)
AB164	0.87 (I)	0.89 (I)	1.38 (I)	0.62 (I)	0.48 (S)
AB167	1.10 (I)	0.62 (I)	0.94 (I)	0.85 (I)	0.37 (S)
AB198	0.94 (I)	0.52 (I)	0.85 (I)	0.75 (I)	0.28 (S)
AB307	0.71 (I)	0.99 (I)	1.08 (I)	1.27 (I)	1.04 (I)
AB313	0.75 (I)	0.71 (I)	0.49 (S)	0.51 (I)	0.47 (S)
AB315	0.92 (I)	1.04 (I)	1.08 (I)	0.92 (I)	1.08 (I)

FICI, fractional inhibitory concentration index; ≤ 0.5 , synergistic, S; $> 0.5-4.0$, indifferent, I; and > 4 , antagonistic effect, AT

To date, the best antibiotic monotherapy or combination therapy for treating this pathogen is still unknown. *In vitro* studies can be carried out to help clinical decisions regarding the most appropriate therapeutic regimen.

Fosfomycin, a phosphonic acid derivative, inhibits bacterial cell wall synthesis and has activity against gram-positive bacteria (eg, *Staphylococcus* spp, *Streptococcus* spp and *Enterococcus* spp) and gram-negative bacteria, including

IN VITRO ACTIVITY OF ANTIMICROBIAL AGENT COMBINATIONS AGAINST CRAB

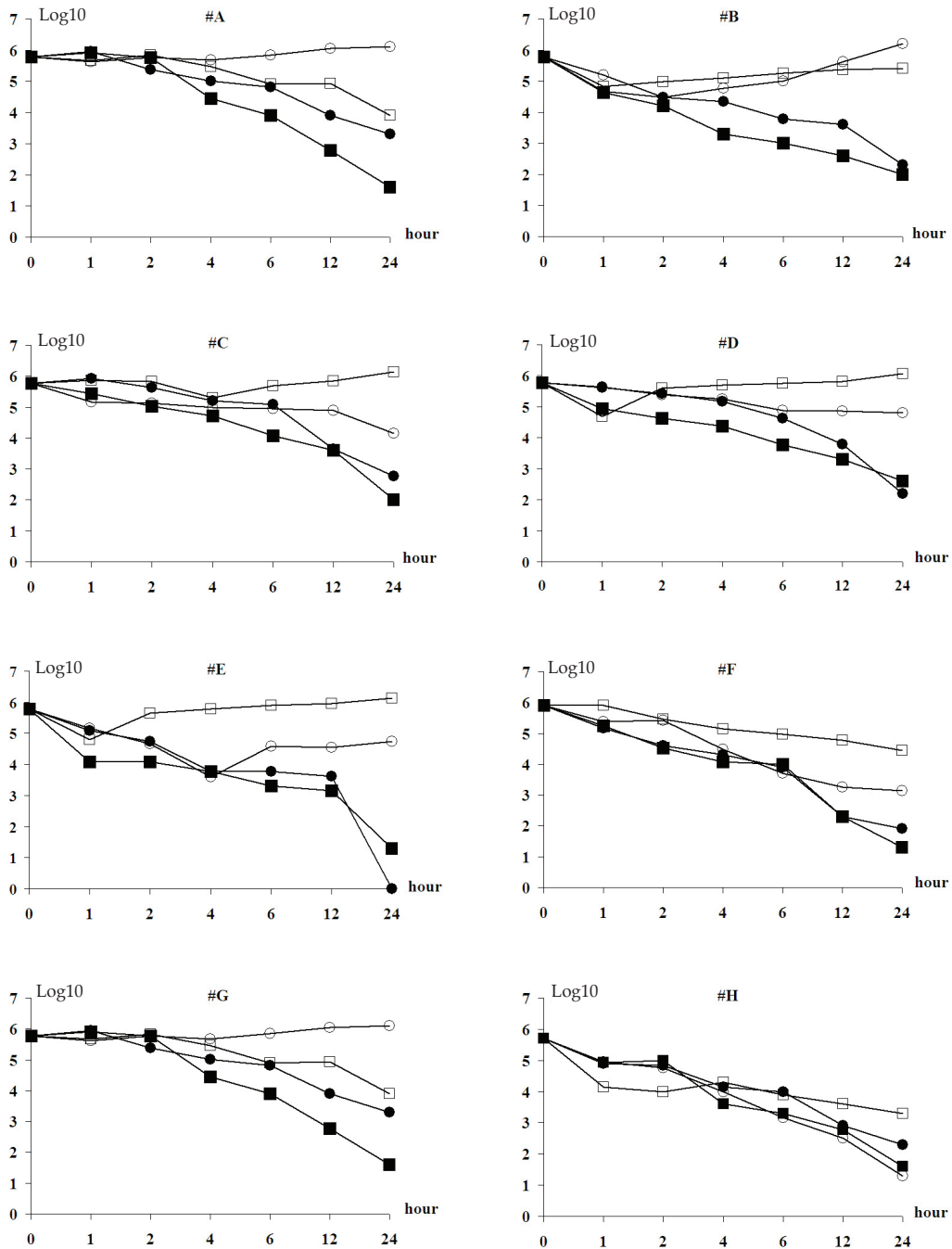


Fig 2–Time-kill curves of synergistic and bactericidal effects of combinations of antimicrobial agents against carbapenem-resistant *A. baumannii* clinical isolates: AB23 (A), AB54 (B), AB164 (C), AB167 (D), AB198 (E), AB307 (F), AB 313 (G), and AB315 (H). Open circles, sulbactam at a concentration of 1xMIC; filled squares, sulbactam at 1xMIC + fosfomycin at 1xMIC; filled circles, sulbactam at 1xMIC + fosfomycin at 1/4 x MIC; open squares, fosfomycin at 1xMIC.

ESBL-producing microorganisms (Falagas *et al*, 2008b). Falagas *et al* (2008a) reported fosfomycin MIC against 30 *A. baumannii* strains ranged from 64 to >512 mg/l (MIC₅₀: 256 mg/l; MIC₉₀: >512 mg/l). In our study, the fosfomycin MIC ranged from 128-512 mg/l (MIC₅₀: 128; MIC₉₀: 256 mg/l). The MIC of imipenem against the 8 strains of MBL-positive CRAB ranged from 16-64 mg/l. Of all the antibiotic combinations studied, the combination of sulbactam and fosfomycin showed the best synergistic effect and bactericidal effect using the time kill-method. Concentrations of these agents in our study were within the therapeutic range. With pharmacokinetic studies, the doses of fosfomycin of 2 g and 8 g IV gave maximum blood concentrations of 145 mg/l and 692 mg/l, respectively. These concentrations were well above the concentrations used in our study (Bando and Toyoshima, 1984; Sauermann *et al*, 2005). The use of fosfomycin with sulbactam may be possible in clinical practice. A previous study by Martinez-Martinez *et al* (1996) showed the synergy of fosfomycin with amikacin or tobramycin were 44% and 32%, respectively, against 34 isolates of MDR-AB. Our study adds to these previous findings by demonstrating a synergistic effect between sulbactam and fosfomycin against CRAB. We found no synergism between imipenem and sulbactam, unlike the findings of Song *et al* (2007) who found this combination had synergistic activity at 1 x MIC. No synergism was seen in our study for the combinations of colistin and sulbactam, and colistin and imipenem.

Our study had certain limitations: 1) we identified carbapenemase enzymes, which are the most common mechanisms in MDR-AB, but we did not study other mechanisms of drug resistance, such as loss of porins, efflux pump, or changing

of PBP, thus, we could not explain why the susceptibility of organisms in our setting differed from other setting; 2) the exact synergistic mechanism of fosfomycin with sulbactam was not clear in our study. We hypothesize fosfomycin likely inhibits pyruvyl transferase responsible for transformation of N-acetylglucosamine into N-acetylmuramic acid which is the first step for cell wall synthesis (Greenwood, 1994). β -lactam antibiotics inhibit the latter step of this process. Therefore the inhibition of the same process of peptidoglycan synthesis, with different target mechanisms of action may explain the synergistic effect of these two antibiotics; 3) even though synergism between fosfomycin and sulbactam was clearly demonstrated in our *in vitro* study, there were no clinical studies to confirm this benefit, *in vivo*. Further studies are needed to determine this.

Sulbactam and fosfomycin had a synergistic bactericidal effect against CRAB isolates producing OXA-23 carbapenemase in our study. However, the use of combination antimicrobial regimens need to be tested in the clinical settings because differences in the epidemiology of resistant genes may influence the efficacy of antibiotic combination treatment.

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