Individual or Combined Effects of Meropenem, Imipenem, Sulbactam, Colistin, and Tigecycline on Biofilm-Embedded *Acinetobacter baumannii* and Biofilm Architecture

Yung-Chih Wang,^{a,b} Shu-Chen Kuo,^c Ya-Sung Yang,^a Yi-Tzu Lee,^d Chun-Hsiang Chiu,^{a,b} Ming-Fen Chuang,^e Jung-Chung Lin,^a Feng-Yee Chang,^a Te-Li Chen^{b,f}

Division of Infectious Diseases and Tropical Medicine, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan^a; Institute of Clinical Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan^b; National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Miaoli County, Taiwan^c; Emergency Department, Taipei Veterans General Hospital, Taipei, Taiwan^d; Division of Infectious Diseases, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan^e; Graduate Institute of Life Science, National Defense Medical Center, Taipei, Taiwan^f

Acinetobacter baumannii biofilms are difficult to eradicate. We investigated the effects of meropenem (2 mg/liter), imipenem (2 mg/liter), sulbactam (4 mg/liter), colistin (2 mg/liter), and tigecycline (2 mg/liter), alone or in combination, on biofilm-embedded carbapenem-resistant and carbapenem-susceptible *A. baumannii* (CRAb and CSAb, respectively) cells, as well as on the architecture of the biofilms. *A. baumannii* ATCC 15151 (Ab15151) and its OXA-82-overproducing transformant, along with two clinical CSAb and two clinical CRAb isolates of differing clonalities, were used. The minimal bactericidal concentrations for biofilm-embedded cells of the six tested isolates were >50-fold those of their planktonic cells. When used individually, meropenem exhibited a higher killing effect than the other four antimicrobials on biofilm-embedded CSAb cells in the colony biofilm assay. For two clinical CRAb isolates, meropenem plus sulbactam or sulbactam plus tigecycline showed >100-fold the bactericidal effect exhibited by these agents used alone after 48 h of treatment. The effect of antimicrobials on the architecture of Ab15151 biofilm emitting green fluorescence was determined by confocal laser scanning microscopy using COMSTAT software. Significant decreases in the maximum biofilm thickness were observed after exposure to meropenem and imipenem. Meropenem plus sulbactam significantly decreased the biomass and mean thickness and increased the roughness coefficient of biofilms, but sulbactam plus tigecycline only decreased the maximum and mean biofilm thickness compared to any of these agents used alone. Meropenem was active against biofilm-embedded CSAb, whereas meropenem plus sulbactam exhibited synergism against biofilm-embedded CRAbs and caused significantly more damage to the biofilm architecture than did any of the agents used alone.

Biofilm formation is a strategy used by bacteria for the colonization of different environments, thus promoting persistent infections (1, 2). Biofilm-embedded cells are highly resistant to antimicrobials and are therefore difficult to eliminate (2–5). Several mechanisms are involved in biofilm formation, accounting for the difficulties in managing biofilm-associated infections (2–5). The stationary-phase physiology and extracellular matrix components contribute to the antimicrobial resistance of bacterial biofilms; the interplay of penetration of antimicrobials into biofilm and bacterial resistance mechanisms, such as β -lactamase and efflux pumps, determine the killing effect of antimicrobials (2–5). Numerous antimicrobial regimens either with a single agent or combinations have been proposed to treat biofilm-associated infections (6–9).

Acinetobacter baumannii is equipped with multiple resistance mechanisms and is a well-known pathogen in health care-associated infections. In device-related infections, biofilm development increases the difficulties in eradicating drug-resistant *A. baumannii* (10). However, reports on the management of biofilm-embedded *A. baumannii* strains are scarce (1, 11, 12). Antimicrobial therapies for patients with carbapenem-resistant *A. baumannii* (CRAb) infections differ from those with carbapenem-susceptible *A. baumannii* (CSAb) infections (13–15). However, it is not known whether different treatment regimens should be used for CRAb and CSAb biofilms. Given the lack of novel antimicrobials available in the clinical setting, we investigated the effects of meropenem, imipenem, sulbactam, colistin, and tigecycline alone or in

combination on biofilm-embedded CRAb and CSAb strains. Their influence on the architecture of *A. baumannii* biofilms was also examined.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. The reference strains *A. baumannii* ATCC 15151 (Ab15151) and ATCC 17978 (Ab17978), and six randomly selected clinical isolates of CSAb were used for the detection of biofilm-forming capability. To determine the impact of carbapenem resistance on biofilm formation and the effect of treatment, six randomly selected clinical isolates of CRAb with different pulsotypes were also included. Ab15151 carrying *bla*_{OXA-82} and the promoter region in the upstream insertion sequence ISAbal [Ab15151(pOXA-82-2)] (16) was in-

Received 11 March 2016 Returned for modification 1 April 2016

Accepted 12 May 2016

Accepted manuscript posted online 23 May 2016

Citation Wang Y-C, Kuo S-C, Yang Y-S, Lee Y-T, Chiu C-H, Chuang M-F, Lin J-C, Chang F-Y, Chen T-L. 2016. Individual or combined effects of meropenem, imipenem, sulbactam, colistin, and tigecycline on biofilm-embedded *Acinetobacter baumannii* and biofilm architecture. Antimicrob Agents Chemother 60:4670–4676. doi:10.1128/AAC.00551-16.

Address correspondence to Te-Li Chen, tecklayyy@gmail.com.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00551-16>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

cluded because the plasmid harboring *bla*_{OXA-82} is widespread in CRAB isolates from Taiwan (16).

A fluorescence-labeled strain, Ab15151(pGFP-apra), was constructed to observe the effects of antimicrobials on the architecture of *A. baumannii* biofilms using confocal microscopy. Briefly, GFPmut2 (17) was fused with the cat promoter (18), which was excised, blunted, and cloned into the NruI site of pWH1266 (19), to form the plasmid pWH-PcatGFPmut2. The full-length apramycin resistance gene was also cloned into pWH-PcatGFPmut2 as a selection marker via its EcoRI restriction site. This plasmid was then transformed into Ab15151 (see Fig. S1 in the supplemental material). The generation of electrocompetent cells and electrotransformation were performed as previously described (20). Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA).

Antimicrobials. Meropenem, imipenem, sulbactam, colistin, and tigecycline were used alone or in combination for biofilm treatments. The concentrations of all antimicrobials except tigecycline were adjusted to the susceptibility breakpoint concentrations (SBCs) recommended by the Clinical and Laboratory Standards Institute (CLSI) (21). Specifically, the following concentrations were used: meropenem, 2 mg/liter; imipenem, 2 mg/liter; sulbactam, 4 mg/liter; and colistin, 2 mg/liter. For tigecycline, the U.S. Food and Drug Administration (FDA)-recommended susceptibility interpretation criterion for *Enterobacteriaceae* (2 mg/liter) was used (22). All antimicrobials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Biofilm formation and measurement. The bacterial strains were cultured for 1 day at 37°C in 5 ml of Luria-Bertani (LB) broth supplemented with 1% D-glucose (LBglu). The cultures were diluted in LBglu to an optical density at 600 nm (OD₆₀₀) of 0.03, and 200 µl of the final solution was added to each well of a 96-well tissue culture polystyrene plate. The cultures were subsequently grown with shaking (180 rpm) for 24 h and 48 h at 30°C. The suspensions were removed, the wells were washed with phosphate-buffered saline (PBS), and 200 µl of 0.1% crystal violet in H₂O was added to stain the cells (23). The plates were then incubated for 20 min with gentle agitation, thoroughly washed with PBS, and the stained biofilms were solubilized with 200 µl of 95% ethanol for 10 min with gentle agitation. The amount of biofilm formed was quantified by measuring the optical density at 595 nm (OD₅₉₅).

MIC and minimal bactericidal concentrations for biofilm-embedded and planktonic cells. An initial inoculum of 5×10^5 CFU/ml was used to determine the MIC and minimal bactericidal concentration for biofilm-embedded cells (MBC-B) and planktonic cells (MBC-P). The MICs of *A. baumannii* were determined by the broth microdilution method, according to CLSI guidelines (24). Tests were performed in triplicate for each antimicrobial. The MBC was determined as the antibiotic concentration that reduced the number of viable cells by $\geq 99.9\%$ using colony counts according to the CLSI guidelines (25). To determine the MBC-P, *A. baumannii* cells were transferred into the wells of a 96-well microtiter dish, and serially diluted antimicrobials were added. After 24 h of antimicrobial exposure, bacterial survival was assessed by plating 2 µl of diluted culture onto LB agar plates. The procedure to determine the MBC-B was modified as previously described (26–28). Briefly, biofilm formation was allowed for 24 h. Serial dilutions of antimicrobials were added to the wells and incubated for an additional 24 h. The antimicrobial-containing medium was replaced with fresh medium. After further incubation for 24 h, the bacteria that detached from the biofilm and survived the antimicrobial treatment were transferred onto LB agar to determine the MBC-B.

Colony biofilm assay to determine antimicrobial activity on biofilm-embedded cells. The colony biofilm assay was performed in 96-well culture plates with a starting inoculum of 2×10^5 CFU/ml, as described previously (7, 29). After 24 h of incubation, the medium in the wells was removed by aspiration. The biofilms were treated with an antimicrobial alone or in combination for 24 h at 37°C (24-h treatment group). The antimicrobial-containing medium was then gently aspirated and replaced with fresh medium containing antimicrobials. The biofilm was then in-

cubated further for 24 h at 37°C (48-h treatment group). The biofilm surfaces were scratched with a 10-µl blue inoculation loop after 24 h and 48 h of incubation, and the cells were suspended in culture medium. The samples were serially diluted and plated for viable cell counting after overnight culture at 37°C. All the experiments were performed in triplicate and repeated on three separate occasions. For single agents, a $\geq 1,000$ -fold decrease in bacterial load was defined as the presence of killing effect. For combination therapy, synergism was defined as a ≥ 100 -fold decrease in bacterial load after treatment with a combination regimen in comparison to that with the most active antimicrobial in the combination used alone.

Microscopy and image analysis of architecture of biofilms. Confocal laser scanning microscopy (CLSM) was used to observe the biofilm architecture (30). Aliquots (100 µl) of overnight cultures of Ab15151(pGFP-apra) were added to 50-ml centrifuge tubes containing 10 ml of 1% LBglu supplemented with 50 mg/liter of apramycin. Glass coverslips were partially immersed in the solution over a 24-h period to allow biofilm formation, as previously described (31). At 24 h after inoculation, the spent culture was replaced with fresh medium, and the antimicrobials were added. After 24 h of antimicrobial treatment, the antimicrobial-containing medium was then aspirated and replaced with fresh medium containing antimicrobials. The biofilms were allowed to form for another 24 h.

To visualize green fluorescent protein (GFP), all observations and acquisitions were performed on an Olympus FluoView FV1000 CLSM (Olympus, USA). At least 4 independent experiments were carried out. Images were digitally reconstructed using the MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA).

Biofilm structure was quantified using confocal z-stacks and the image analysis software package COMSTAT (Technical University of Denmark, Lyngby, Denmark) (32). In this study, parameters, such as biomass, maximum biofilm thickness, mean biofilm thickness, and roughness coefficient, were compared between the biofilms formed, before and after antimicrobial treatment, and between the single-agent and combination treatment groups. A rough biofilm shows varying thicknesses, whereas a smooth biofilm consists of a more homogenous flat layer of cells. An increase in roughness coefficient suggests structural changes and thus indicates a greater disruption of biofilm structure.

Statistical analysis. The parameters obtained from COMSTAT were analyzed using the Student *t* test. A *P* value of < 0.05 was considered statistically significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 19.0 (SPSS, Chicago, IL, USA).

RESULTS

Biofilm formation of *A. baumannii* isolates and minimal inhibitory and bactericidal concentrations of antimicrobials. Table S2 in the supplemental material presents the MICs of meropenem, imipenem, sulbactam, colistin, and tigecycline for all the tested isolates. Biofilm formation of Ab17978, Ab15151, Ab15151(pOXA-82-2), six clinical CSAb isolates, and six clinical CRAB clinical isolates are shown in Fig. S2 in the supplemental material. The reference strain Ab15151 and Ab15151(pOXA-82-2) were selected instead of Ab17978 for the following experiments because of their stronger biofilm-forming capacity. Two CSAb (Ab1770 and Ab2075) and two CRAB (Ab1987 and Ab2147) isolates with stronger biofilm formation potential were also included. The dramatic increase in the MBCs of different antimicrobials for biofilm cells compared to those for planktonic cells of the six tested isolates is shown in Table 1. The MBC-Bs of all five antimicrobials were > 50 -fold the MBC-Ps.

Antibacterial efficacies of antimicrobials alone or in combination against biofilm-embedded CRAB and CSAb cells. We then determined the killing effect of these antimicrobials on biofilm-embedded cells by treatment with each antimicrobial at the SBC for 24 and 48 h (Tables 2 and 3; see also Fig. S3 in the supple-

TABLE 1 MBCs of antimicrobials against biofilm-embedded and planktonic cells of *Acinetobacter baumannii* isolates^a

Antimicrobial ^b	Ab15151		Ab1770		Ab2075		Ab15151 (pOXA-82-2)		Ab1987		Ab2147	
	MBC-P	MBC-B	MBC-P	MBC-B	MBC-P	MBC-B	MBC-P	MBC-B	MBC-P	MBC-B	MBC-P	MBC-B
MEM	4	400	4	>3,200	4	>3,200	32	3,200	64	>3,200	64	>3,200
IPM	2	800	1	>3,200	4	>3,200	8	>3,200	64	>3,200	64	>3,200
SUL	64	>3,200	4	>3,200	16	>3,200	64	>3,200	64	>3,200	64	>3,200
CST	4	>200	2	200	2	>200	4	>200	4	>200	2	200
TGC	32	>1,600	1	200	1	>1,600	32	>1,600	32	>1,600	32	>1,600

^a Values are presented in mg/liter and represent the mode of at least 6 biological replicates. The exact MBC-B of some antimicrobials could not be determined due to the insolubility of the antimicrobials above the concentration used.

^b MEM, meropenem; IPM, imipenem; SUL, sulbactam; CST, colistin; TGC, tigecycline.

mental material). At 24 h, all antimicrobials failed to exhibit a killing effect against CSAb and CRAb cells. None of the combination regimens showed synergism.

At 48 h, only meropenem exerted an obvious killing effect on the three tested CSAb isolates but not CRAb isolates. The killing effect of meropenem against CSAb isolates exhibited a 3.30- to 5.33-log CFU/ml reduction in bacterial load. Imipenem was the second-most-active agent against the CSAb isolates. Imipenem and tigecycline exerted a killing effect on the reference strain Ab15151. For CRAb, even though carbapenems were generally ineffective, the combination of meropenem plus sulbactam exerted synergism on both clinical CRAb isolates. A borderline synergism was also observed (a reduction of 1.81 log CFU/ml) for Ab15151(pOXA-82-2). However, the addition of sulbactam to meropenem provided no benefit in the case of any CSAb isolates. At 48 h, imipenem plus tigecycline showed synergism in one clinical CSAb isolate. One clinical CSAb and two clinical CRAb isolates treated with sulbactam plus tigecycline showed a >100-fold reduction in bacterial load compared with the load observed following treatment of these isolates with either antimicrobial alone.

Microscopy and image analysis of biofilm architecture. To

determine the effect of antimicrobials on biofilm architecture, we observed the structure of Ab15151(pGFP-apra) biofilms using confocal microscopy (Fig. 1). The MICs, MBC-Ps, and MBC-Bs of the five tested antimicrobials against Ab15151(pGFP-apra) were the same as those of its parent strain Ab15151 (data not shown). Meropenem and imipenem resulted in larger and more-rounded biofilm cells (Fig. 1b and c). On the contrary, sulbactam-treated biofilm cells displayed a filamentous change (Fig. 1d). As shown in Table 4, COMSTAT analysis revealed a significant decrease in the maximum thickness of the biofilms after exposure to meropenem and imipenem. No significant change in the *A. baumannii* biofilm architecture was found upon treatment with the other antimicrobials.

Although meropenem plus sulbactam and sulbactam plus tigecycline either had no or occasional synergism against biofilm-embedded CSAb, these combinations were more potent to disrupt the CSAb biofilm architecture than any of the antimicrobials used alone. CLSM images showed relatively loose biofilm structures in combination-treated cells compared to those exposed to single antimicrobials (Fig. 1g and h). The meropenem-plus-sulbactam group differed significantly ($P < 0.05$) from the sulbactam-

TABLE 2 Bacterial load of three carbapenem-susceptible *Acinetobacter baumannii* strains in biofilms after 24 h and 48 h of exposure to five antimicrobials used alone or in combination at their susceptibility breakpoint concentrations^a

Antimicrobial(s)	Bacterial load (log CFU/ml) in biofilms by strain after:					
	24 h			48 h		
	Ab15151	Ab1770	Ab2075	Ab15151	Ab1770	Ab2075
No antimicrobial	7.66 ± 0.40	7.41 ± 0.10	7.53 ± 0.11	8.14 ± 0.23	8.06 ± 0.17	7.74 ± 0.07
MEM	6.33 ± 0.84	6.84 ± 0.19	6.12 ± 0.27	2.81 ± 0.26 ^b	4.76 ± 0.51 ^b	3.60 ± 0.19 ^b
IPM	5.42 ± 0.50	6.94 ± 0.32	6.17 ± 0.21	3.97 ± 0.72 ^b	7.81 ± 0.20	5.37 ± 0.92
SUL	8.07 ± 0.97	7.29 ± 0.08	6.65 ± 0.18	6.64 ± 0.25	6.26 ± 0.27	6.05 ± 0.18
CST	8.41 ± 0.47	7.57 ± 0.16	8.13 ± 0.23	7.30 ± 0.54	8.74 ± 0.08	7.79 ± 0.34
TGC	5.81 ± 0.67	7.58 ± 0.20	7.33 ± 0.09	3.80 ± 0.13 ^b	7.38 ± 0.16	7.45 ± 0.18
MEM + SUL	5.61 ± 0.59	6.26 ± 0.63	6.10 ± 0.14	2.56 ± 0.35	4.96 ± 0.35	3.53 ± 0.07
MEM + CST	5.74 ± 0.23	6.37 ± 0.35	4.99 ± 0.29	3.57 ± 0.69	5.06 ± 0.48	3.80 ± 0.31
MEM + TGC	5.11 ± 0.47	6.63 ± 0.11	5.89 ± 0.31	2.99 ± 0.39	4.46 ± 0.22	3.19 ± 0.70
IPM + SUL	5.73 ± 0.19	6.60 ± 0.09	6.10 ± 0.20	4.05 ± 0.18	4.88 ± 0.25	4.53 ± 0.33
IPM + CST	5.64 ± 0.26	6.66 ± 0.18	5.56 ± 0.48	4.23 ± 0.41	7.98 ± 0.25	5.29 ± 1.28
IPM + TGC	5.33 ± 0.34	6.63 ± 0.12	4.51 ± 0.38	3.58 ± 0.92	4.74 ± 0.27 ^c	3.68 ± 0.72
SUL + CST	7.34 ± 0.05	7.76 ± 0.22	6.68 ± 0.10	6.19 ± 0.33	6.73 ± 0.43	6.25 ± 0.42
SUL + TGC	5.47 ± 0.53	7.16 ± 0.15	5.98 ± 0.13	4.26 ± 0.38	6.01 ± 0.14	3.22 ± 0.22 ^c
CST + TGC	5.28 ± 0.67	7.20 ± 0.18	7.51 ± 0.27	3.95 ± 0.21	7.74 ± 0.15	7.45 ± 0.41

^a Susceptibility breakpoint concentrations of antimicrobials: meropenem (MEM), 2 mg/liter; imipenem (IPM), 2 mg/liter; sulbactam (SUL), 4 mg/liter; colistin (CST), 2 mg/liter; and tigecycline (TGC), 2 mg/liter. Values are means ± standard deviations.

^b Indicates bactericidal effect (>3-log reduction in bacterial load compared to that without antibiotic treatment).

^c Indicates synergism.

TABLE 3 Bacterial load (log CFU/ml) of three carbapenem-resistant *Acinetobacter baumannii* strains in biofilms after 24 h and 48 h of exposure to five antimicrobials used alone or in combination at susceptibility breakpoint concentrations^a

Antimicrobial(s)	Bacterial load (log CFU/ml) in biofilms by strain after:					
	24 h			48 h		
	Ab15151 (pOXA-82-2)	Ab1987	Ab2147	Ab15151 (pOXA-82-2)	Ab1987	Ab2147
No antimicrobial	8.20 ± 0.61	6.93 ± 0.04	7.15 ± 0.09	9.35 ± 0.19	7.48 ± 0.22	7.76 ± 0.19
MEM	6.95 ± 0.42	7.09 ± 0.39	7.16 ± 0.26	7.94 ± 0.18	7.08 ± 0.18	7.07 ± 0.19
IPM	8.01 ± 0.34	7.06 ± 0.09	7.20 ± 0.11	9.33 ± 0.43	7.81 ± 0.16	8.06 ± 0.10
SUL	8.21 ± 1.23	7.45 ± 0.30	7.48 ± 0.21	6.59 ± 0.23	7.11 ± 0.21	6.65 ± 0.25
CST	8.56 ± 0.52	7.62 ± 0.04	7.89 ± 0.16	7.40 ± 0.54	9.06 ± 0.11	9.07 ± 0.36
TGC	6.77 ± 0.29	7.70 ± 0.21	7.85 ± 0.52	5.00 ± 1.03 ^b	8.87 ± 0.11	9.41 ± 0.20
MEM + SUL	6.56 ± 0.14	6.10 ± 0.09	6.33 ± 0.12	4.78 ± 0.40	4.57 ± 0.49 ^c	4.11 ± 0.37 ^c
MEM + CST	7.20 ± 0.20	6.59 ± 0.28	6.99 ± 0.70	7.93 ± 0.13	7.79 ± 0.39	6.09 ± 0.64
MEM + TGC	5.72 ± 0.48	6.01 ± 0.17	6.37 ± 0.34	4.37 ± 0.31	7.27 ± 0.24	6.33 ± 0.53
IPM + SUL	7.25 ± 0.10	6.40 ± 0.07	6.48 ± 0.11	6.87 ± 0.69	5.57 ± 0.61	5.21 ± 0.26
IPM + CST	7.89 ± 0.38	7.47 ± 0.06	7.52 ± 0.07	9.27 ± 0.54	8.20 ± 0.29	8.60 ± 0.17
IPM + TGC	7.51 ± 0.74	6.32 ± 0.20	6.72 ± 0.18	5.71 ± 1.15	7.95 ± 0.08	8.28 ± 0.23
SUL + CST	7.34 ± 0.23	7.65 ± 0.52	7.61 ± 0.38	6.62 ± 0.53	6.42 ± 0.13	6.01 ± 0.41
SUL + TGC	6.10 ± 0.32	7.68 ± 0.31	7.64 ± 0.50	4.06 ± 0.38	4.92 ± 0.37 ^c	3.94 ± 0.30 ^c
CST + TGC	6.54 ± 0.35	7.69 ± 0.08	7.99 ± 0.30	4.92 ± 0.08	8.50 ± 0.16	9.40 ± 0.20

^a Susceptibility breakpoint concentrations of antimicrobials: meropenem (MEM), 2 mg/liter; imipenem (IPM), 2 mg/liter; sulbactam (SUL), 4 mg/liter; colistin (CST), 2 mg/liter; and tigecycline (TGC), 2 mg/liter. Values are means ± standard deviations.

^b Indicates bactericidal effect (>3-log reduction in bacterial load compared to that without antibiotic treatment).

^c Indicates synergism.

only or meropenem-only group in three of the four tested parameters, i.e., the biomass, mean thickness, and roughness coefficient (Table 4). The sulbactam-plus-tigecycline group showed significant ($P < 0.05$) differences compared to the sulbactam-only or tigecycline-only group in maximum and mean thickness only (Table 4).

DISCUSSION

The present study demonstrated that the effectiveness of antimicrobial treatments differed between CRAb and CSAb biofilms. Meropenem had the greatest activity against biofilm-embedded CSAb cells but had no effect on biofilm-embedded CRAb cells.

FIG 1 Confocal laser scanning microscopy (CLSM) images of 24-h-old biofilms of *Acinetobacter baumannii* ATCC 15151(pGFP-apra) exposed to antimicrobials at serum-susceptible concentrations for 48 h. A representative CLSM image is shown for each sample. Biofilms exposed to no antimicrobials (a), meropenem (b), imipenem (c), sulbactam (d), colistin (e), tigecycline (f), meropenem plus sulbactam (g), and sulbactam plus tigecycline (h). The susceptibility breakpoint concentrations of antimicrobials were meropenem, 2 mg/liter; imipenem, 2 mg/liter; sulbactam, 4 mg/liter; colistin, 2 mg/liter, and tigecycline, 2 mg/liter.

TABLE 4 COMSTAT analysis of z stacks from 24-h-old biofilms of *Acinetobacter baumannii* ATCC 15151(pGFP-apra) on glass coverslips exposed to antimicrobials at susceptibility breakpoint concentrations^a

Antimicrobial(s)	Biomass (mm ³ /mm ²)	Maximum thickness (mm)	Mean thickness (mm)	Roughness coefficient
No antimicrobial	17.39 ± 4.50	40.52 ± 1.26	23.62 ± 5.61	0.36 ± 0.19
MEM	19.90 ± 6.63	36.84 ± 1.44 ^b	26.92 ± 5.13	0.25 ± 0.18
IPM	17.20 ± 9.21	35.71 ± 2.42 ^b	23.07 ± 9.60	0.34 ± 0.22
SUL	16.61 ± 8.20	40.89 ± 2.85	25.38 ± 6.97	0.43 ± 0.24
CST	17.18 ± 4.70	38.47 ± 3.21	22.87 ± 4.21	0.26 ± 0.12
TGC	16.90 ± 5.48	38.07 ± 3.19	22.63 ± 5.99	0.39 ± 0.18
MEM + SUL ^c	8.32 ± 4.84 ^b	34.92 ± 3.33 ^b	13.70 ± 6.69 ^b	0.73 ± 0.28 ^b
SUL + TGC ^d	12.40 ± 4.50 ^b	32.55 ± 2.58 ^b	16.50 ± 3.84 ^b	0.52 ± 0.19

^a All results are an average of the results from 8 image stacks acquired in 4 separate experiments. Values are mean ± standard deviation. Susceptibility breakpoint concentrations of antimicrobials were: meropenem (MEM), 2 mg/liter; imipenem (IPM), 2 mg/liter; sulbactam (SUL), 4 mg/liter; colistin (CST), 2 mg/liter; and tigecycline (TGC), 2 mg/liter.

^b $P < 0.05$ for the group with antimicrobials versus the group without antimicrobials.

^c The meropenem-plus-sulbactam group differed significantly ($P < 0.05$) from the sulbactam-only group in all four parameters and from the meropenem-only group in biomass, mean thickness, and roughness coefficient, but not in maximum thickness.

^d The sulbactam-plus-tigecycline group differed significantly ($P < 0.05$) from the sulbactam-only group and from the tigecycline-only group in maximum thickness and mean thickness.

Meropenem plus sulbactam showed synergism with the two clinical CRAB isolates, whereas the combination of sulbactam plus tigecycline showed synergism with the two CRAB isolates and one CSAB isolate. Compared to sulbactam plus tigecycline, meropenem plus sulbactam caused significantly more damage to the architecture of *A. baumannii* biofilms.

Our study and others have illustrated better activities of carbapenems than other antimicrobials against biofilm-embedded cells (11, 29, 33) and biofilm architecture (8, 34). Since the biofilm is a complex community, bacterial cells growing in biofilms are physiologically heterogeneous. The bacterial cells in the outer layer of biofilms are active and continue to replicate (35–38). In our study, bacterial cells showed obvious morphological changes after exposure to carbapenems. In previous studies, meropenem was found to induce filamentous changes in the bacteria at lower concentrations and the formation of spheroplasts at higher concentrations (33, 39, 40). Imipenem-treated bacteria were observed to change into large ovoid cells (11, 33, 34, 39). The effect of carbapenems on bacterial morphology may be correlated with their affinities for different penicillin-binding proteins (PBPs) (33, 39–41). This observation implies that carbapenems might kill growing and dividing bacterial cells in certain parts of the biofilm through the inhibition of PBPs.

The killing effect of carbapenems on biofilm-embedded bacteria has been associated with the disruption of biofilm architecture for *Haemophilus influenzae* (8) and *Klebsiella pneumoniae* (34), as seen in our findings. However, there are very few studies that address how the killing of bacteria alters the architecture of the established biofilms. The extracellular matrix of a biofilm is a mixture of extracellular DNA (eDNA), lipids, polysaccharides, and extracellular proteins that provide architectural structure and mechanical stability to the attached bacterial population (42–45). Many of the proteins in the extracellular matrix have been revealed to polymerize into higher-order structures and anchor to bacterial cells through some proteins (37). Killing the cells in the outermost layer of biofilms may interrupt the connection between bacterial cells and biofilm-anchoring proteins and then disrupt the biofilm architecture. The eDNA is also involved in biofilm formation and cohesion of biofilms to provide mechanical stability (42–44). The eDNA in the matrix has been found to increase

resistance to cationic antimicrobial peptides and aminoglycosides but not to β -lactams (46). This feature may contribute to the antibiofilm activity of carbapenems. Recently, imipenem has been proposed to show obvious antibiofilm effects by reducing the amount of eDNA (47). The lack of eDNA may hinder biofilm formation and architecture (44).

In our study, carbapenems, especially meropenem, were effective against biofilm-embedded CSAB at a clinically achievable level. The poor effects exhibited by other antimicrobials in our study may be attributed to the use of concentrations that were lower than those used in previous studies (11, 12). In a previous study, higher concentrations (>8-fold the MIC) of sulbactam were required to increase its activity against *A. baumannii* biofilms formed on coverslips (11). Colistin at 400-fold the MIC expressed excellent activity against biofilm-embedded *A. baumannii* cells (12). Their unachievable concentrations in human serum render these antimicrobials as undesirable options for systemic use in *A. baumannii* biofilm-associated infections.

Since the effect of β -lactams might be hampered by the presence of β -lactamase in the biofilm matrix (3, 48, 49), it is not surprising that carbapenems were generally ineffective against biofilm-embedded CRAB isolates in our study. Combination therapies have been used to treat CRAB infections (13–15). In addition, several studies have demonstrated the use of combination treatments for biofilms formed by *S. aureus* (6, 9), *Pseudomonas aeruginosa* (29, 48), and *Enterococcus* species (7). In this study, the combination of meropenem plus sulbactam and sulbactam plus tigecycline showed superior killing effects against CRAB compared to those of any of these antimicrobials used alone. For CSAB, although these combinations did not significantly reduce bacterial cell load compared to that with any agent used alone, they caused significantly more damage to biofilm architecture than did these agents used alone. The clinical benefit of the combination treatment against biofilm architecture should be further confirmed clinically. The superior antibiofilm activity of the combination treatment might be a result of targeting multiple mechanisms. However, the exact mechanism involved in the combination treatment against biofilm cells remains elusive.

Increased resistance to antimicrobials makes the eradication of *A. baumannii* biofilms notoriously difficult. The ≥ 50 -fold in-

crease in MBC-B compared to the MBC-P observed here is consistent with observations from previous studies (2, 3). However, the exact MBC-B of some antimicrobials could not be determined due to the insolubility of the antimicrobials at higher concentrations; therefore, we are unable to compare the effect of carbapenemase on the increase in carbapenem MBC. β -Lactamase contained in the extracellular matrix of biofilms attenuates the activities of β -lactam antibiotics, as described in previous studies (3, 48, 49). Our study showed a better killing effect of carbapenems in CSAb than in CRAb. These results posit that the presence of carbapenemase may affect the MBCs of biofilm-embedded bacteria.

In summary, this *in vitro* study found that meropenem and the combination of meropenem plus sulbactam using serum-susceptible concentrations showed the best activity against biofilm-embedded CSAb and CRAb cells, respectively. The combination of meropenem plus sulbactam also caused significantly more damage to the architecture of CSAb biofilms than did either agent used alone. Further investigations with a larger panel of isolates along with clinical studies are required to confirm these findings.

ACKNOWLEDGMENTS

This work was supported by grants from the Tri-Service General Hospital (TSGH-C104-117 and TSGH-C105-112) and the Ministry of Science and Technology (MOST-104-2314-B-016-022 and MOST-104-2314-B-010-027-MY3).

We disclose no conflicts of interest.

FUNDING INFORMATION

This work, including the efforts of Yung-Chih Wang, was funded by Tri-Service General Hospital (TSGH-C104-117 and TSGH-C105-112). This work, including the efforts of Yung-Chih Wang, was funded by Ministry of Science and Technology, Taiwan (MOST) (MOST-104-2314-B-016-022). This work, including the efforts of Te-Li Chen, was funded by Ministry of Science and Technology, Taiwan (MOST) (MOST-104-2314-B-010-027-MY3).

REFERENCES

- Vidal R, Dominguez M, Urrutia H, Bello H, Gonzalez G, Garcia A, Zemelman R. 1996. Biofilm formation by *Acinetobacter baumannii*. Microbios 86:49–58.
- Lewis K. 2001. Riddle of biofilm resistance. Antimicrob Agents Chemother 45:999–1007. <http://dx.doi.org/10.1128/AAC.45.4.999-1007.2001>.
- Højby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 35:322–332. <http://dx.doi.org/10.1016/j.ijantimicag.2009.12.011>.
- Olsen I. 2015. Biofilm-specific antibiotic tolerance and resistance. Eur J Clin Microbiol Infect Dis 34:877–886. <http://dx.doi.org/10.1007/s10096-015-2323-z>.
- Mah TF. 2012. Biofilm-specific antibiotic resistance. Future Microbiol 7:1061–1072. <http://dx.doi.org/10.2217/fmb.12.76>.
- Tang HJ, Chen CC, Cheng KC, Toh HS, Su BA, Chiang SR, Ko WC, Chuang YC. 2012. *In vitro* efficacy of fosfomycin-containing regimens against methicillin-resistant *Staphylococcus aureus* in biofilms. J Antimicrob Chemother 67:944–950. <http://dx.doi.org/10.1093/jac/dkr535>.
- Tang HJ, Chen CC, Zhang CC, Su BA, Li CM, Weng TC, Chiang SR, Ko WC, Chuang YC. 2013. *In vitro* efficacy of fosfomycin-based combinations against clinical vancomycin-resistant *Enterococcus* isolates. Diagn Microbiol Infect Dis 77:254–257. <http://dx.doi.org/10.1016/j.diagmicrobio.2013.07.012>.
- Uemura Y, Qin L, Gotoh K, Ohta K, Nakamura K, Watanabe H. 2013. Comparison study of single and concurrent administrations of carbapenem, new quinolone, and macrolide against *in vitro* nontypeable *Haemophilus influenzae* mature biofilms. J Infect Chemother 19:902–908. <http://dx.doi.org/10.1007/s10156-013-0598-5>.
- Rose WE, Poppens PT. 2009. Impact of biofilm on the *in vitro* activity of vancomycin alone and in combination with tigecycline and rifampicin against *Staphylococcus aureus*. J Antimicrob Chemother 63:485–488. <http://dx.doi.org/10.1093/jac/dkn513>.
- Villegas MV, Hartstein AI. 2003. *Acinetobacter* outbreaks, 1977–2000. Infect Control Hosp Epidemiol 24:284–295. <http://dx.doi.org/10.1086/502205>.
- Vidal R, Dominguez M, Urrutia H, Bello H, Garcia A, Gonzalez G, Zemelman R. 1997. Effect of imipenem and sulbactam on sessile cells of *Acinetobacter baumannii* growing in biofilm. Microbios 91:79–87.
- Ozbek B, Mataraci E. 2013. *In vitro* effectiveness of colistin, tigecycline and levofloxacin alone and combined with clarithromycin and/or heparin as lock solutions against embedded *Acinetobacter baumannii* strains. J Antimicrob Chemother 68:827–830. <http://dx.doi.org/10.1093/jac/dks472>.
- Song JY, Lee J, Heo JY, Noh JY, Kim WJ, Cheong HJ, Hwang IS. 2008. Colistin and rifampicin combination in the treatment of ventilator-associated pneumonia caused by carbapenem-resistant *Acinetobacter baumannii*. Int J Antimicrob Agents 32:281–284. <http://dx.doi.org/10.1016/j.ijantimicag.2008.04.013>.
- Durante-Mangoni E, Signoriello G, Andini R, Mattei A, De Cristoforo M, Murino P, Bassetti M, Malacarne P, Petrosillo N, Galdieri N, Mocavero P, Corcione A, Viscoli C, Zarrilli R, Gallo C, Utili R. 2013. Colistin and rifampicin compared with colistin alone for the treatment of serious infections due to extensively drug-resistant *Acinetobacter baumannii*: a multicenter, randomized clinical trial. Clin Infect Dis 57:349–358. <http://dx.doi.org/10.1093/cid/cit253>.
- Viehman JA, Nguyen MH, Doi Y. 2014. Treatment options for carbapenem-resistant and extensively drug-resistant *Acinetobacter baumannii* infections. Drugs 74:1315–1333. <http://dx.doi.org/10.1007/s40265-014-0267-8>.
- Chen TL, Lee YT, Kuo SC, Hsueh PR, Chang FY, Siu LK, Ko WC, Fung CP. 2010. Emergence and distribution of plasmids bearing the *bla*_{OXA-51}-like gene with an upstream *IS*_{Aba1} in carbapenem-resistant *Acinetobacter baumannii* isolates in Taiwan. Antimicrob Agents Chemother 54:4575–4581. <http://dx.doi.org/10.1128/AAC.00764-10>.
- Cormack BP, Valdivia RH, Falkow S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33–38. [http://dx.doi.org/10.1016/0378-1119\(95\)00685-0](http://dx.doi.org/10.1016/0378-1119(95)00685-0).
- van Vliet AH, Wooldridge KG, Ketley JM. 1998. Iron-responsive gene regulation in a *Campylobacter jejuni* *fur* mutant. J Bacteriol 180:5291–5298.
- Hunger M, Schmucker R, Kishan V, Hillen W. 1990. Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoeticus* and its use for *Escherichia coli* shuttle plasmids. Gene 87:45–51. [http://dx.doi.org/10.1016/0378-1119\(90\)90494-C](http://dx.doi.org/10.1016/0378-1119(90)90494-C).
- Chen TL, Chang WC, Kuo SC, Lee YT, Chen CP, Siu LK, Cho WL, Fung CP. 2010. Contribution of a plasmid-borne *bla*_{OXA-58} gene with its hybrid promoter provided by *IS*₁₀₀₆ and an *IS*_{Aba3}-like element to beta-lactam resistance in *Acinetobacter* genomic species 13TU. Antimicrob Agents Chemother 54:3107–3112. <http://dx.doi.org/10.1128/AAC.00128-10>.
- Clinical and Laboratory Standards Institute. 2014. Performance standards for antimicrobial susceptibility testing; 24th informational supplement. CLSI document M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.
- Brown SD, Traczewski MM. 2007. Comparative *in vitro* antimicrobial activity of tigecycline, a new glycylcycline compound, in freshly prepared medium and quality control. J Clin Microbiol 45:2173–2179. <http://dx.doi.org/10.1128/JCM.02351-06>.
- O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. 1999. Genetic approaches to study of biofilms. Methods Enzymol 310:91–109. [http://dx.doi.org/10.1016/S0076-6879\(99\)10008-9](http://dx.doi.org/10.1016/S0076-6879(99)10008-9).
- Clinical and Laboratory Standards Institute. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. CLSI document M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
- Clinical and Laboratory Standards Institute. 1999. Methods for determining bactericidal activity of antimicrobial agents; approved guideline. CLSI document M26-A. Clinical and Laboratory Standards Institute, Wayne, PA.
- Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature 426:306–310. <http://dx.doi.org/10.1038/nature02122>.
- Billings N, Millan M, Caldara M, Rusconi R, Tarasova Y, Stocker R,

- Ribbeck K. 2013. The extracellular matrix component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog* 9:e1003526. <http://dx.doi.org/10.1371/journal.ppat.1003526>.
28. Gillis RJ, White KG, Choi KH, Wagner VE, Schweizer HP, Iglewski BH. 2005. Molecular basis of azithromycin-resistant *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 49:3858–3867. <http://dx.doi.org/10.1128/AAC.49.9.3858-3867.2005>.
 29. Hengzhuang W, Wu H, Ciofu O, Song Z, Hoiby N. 2012. *In vivo* pharmacokinetics/pharmacodynamics of colistin and imipenem in *Pseudomonas aeruginosa* biofilm infection. *Antimicrob Agents Chemother* 56:2683–2690. <http://dx.doi.org/10.1128/AAC.06486-11>.
 30. Lawrence JR, Neu TR. 1999. Confocal laser scanning microscopy for analysis of microbial biofilms. *Methods Enzymol* 310:131–144. [http://dx.doi.org/10.1016/S0076-6879\(99\)10011-9](http://dx.doi.org/10.1016/S0076-6879(99)10011-9).
 31. Choi AH, Slamti L, Avci FY, Pier GB, Maira-Litran T. 2009. The *pgaABCD* locus of *Acinetobacter baumannii* encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. *J Bacteriol* 191:5953–5963. <http://dx.doi.org/10.1128/JB.00647-09>.
 32. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, Molin S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146:2395–2407.
 33. Van Laar TA, Chen T, You T, Leung KP. 2015. Sublethal concentrations of carbapenems alter cell morphology and genomic expression of *Klebsiella pneumoniae* biofilms. *Antimicrob Agents Chemother* 59:1707–1717. <http://dx.doi.org/10.1128/AAC.04581-14>.
 34. Chen P, Seth AK, Abercrombie JJ, Mustoe TA, Leung KP. 2014. Activity of imipenem against *Klebsiella pneumoniae* biofilms *in vitro* and *in vivo*. *Antimicrob Agents Chemother* 58:1208–1213. <http://dx.doi.org/10.1128/AAC.01353-13>.
 35. Werner E, Roe F, Bugnicourt A, Franklin MJ, Heydorn A, Molin S, Pitts B, Stewart PS. 2004. Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* 70:6188–6196. <http://dx.doi.org/10.1128/AEM.70.10.6188-6196.2004>.
 36. Walters MC, III, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* 47:317–323. <http://dx.doi.org/10.1128/AAC.47.1.317-323.2003>.
 37. Hobley L, Harkins C, MacPhee CE, Stanley-Wall NR. 2015. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol Rev* 39:649–669. <http://dx.doi.org/10.1093/femsre/fuv015>.
 38. Davies D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov* 2:114–122. <http://dx.doi.org/10.1038/nrd1008>.
 39. Horii T, Kobayashi M, Sato K, Ichiyama S, Ohta M. 1998. An *in-vitro* study of carbapenem-induced morphological changes and endotoxin release in clinical isolates of Gram-negative bacilli. *J Antimicrob Chemother* 41:435–442. <http://dx.doi.org/10.1093/jac/41.4.435>.
 40. Penwell WF, Shapiro AB, Giacobbe RA, Gu RF, Gao N, Thresher J, McLaughlin RE, Huband MD, DeJonge BL, Ehmann DE, Miller AA. 2015. Molecular mechanisms of sulbactam antibacterial activity and resistance determinants in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 59:1680–1689. <http://dx.doi.org/10.1128/AAC.04808-14>.
 41. Urban C, Go E, Mariano N, Rahal JJ. 1995. Interaction of sulbactam, clavulanic acid and tazobactam with penicillin-binding proteins of imipenem-resistant and -susceptible *Acinetobacter baumannii*. *FEMS Microbiol Lett* 125:193–197. <http://dx.doi.org/10.1111/j.1574-6968.1995.tb07357.x>.
 42. Montanaro L, Poggi A, Visai L, Ravaoli S, Campoccia D, Speziale P, Arciola CR. 2011. Extracellular DNA in biofilms. *Int J Artif Organs* 34:824–831. <http://dx.doi.org/10.5301/ijao.5000051>.
 43. Sahu PK, Iyer PS, Oak AM, Pardesi KR, Chopade BA. 2012. Characterization of eDNA from the clinical strain *Acinetobacter baumannii* AI-IMS 7 and its role in biofilm formation. *ScientificWorldJournal* 2012:973436. <http://dx.doi.org/10.1100/2012/973436>.
 44. Ghafoor A, Hay ID, Rehm BH. 2011. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. *Appl Environ Microbiol* 77:5238–5246. <http://dx.doi.org/10.1128/AEM.00637-11>.
 45. Branda SS, Vik S, Friedman L, Kolter R. 2005. Biofilms: the matrix revisited. *Trends Microbiol* 13:20–26. <http://dx.doi.org/10.1016/j.tim.2004.11.006>.
 46. Mulcahy H, Charron-Mazenod L, Lewenza S. 2008. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog* 4:e1000213. <http://dx.doi.org/10.1371/journal.ppat.1000213>.
 47. Wang Y, Bao W, Guo N, Chen H, Cheng W, Jin K, Shen F, Xu J, Zhang Q, Wang C, An Y, Zhang K, Wang F, Yu L. 2014. Antimicrobial activity of the imipenem/rifampicin combination against clinical isolates of *Acinetobacter baumannii* grown in planktonic and biofilm cultures. *World J Microbiol Biotechnol* 30:3015–3025. <http://dx.doi.org/10.1007/s11274-014-1728-7>.
 48. Hengzhuang W, Ciofu O, Yang L, Wu H, Song Z, Oliver A, Hoiby N. 2013. High β -lactamase levels change the pharmacodynamics of beta-lactam antibiotics in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 57:196–204. <http://dx.doi.org/10.1128/AAC.01393-12>.
 49. Dibdin GH, Assinder SJ, Nichols WW, Lambert PA. 1996. Mathematical model of beta-lactam penetration into a biofilm of *Pseudomonas aeruginosa* while undergoing simultaneous inactivation by released beta-lactamases. *J Antimicrob Chemother* 38:757–769. <http://dx.doi.org/10.1093/jac/38.5.757>.