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In vitro Comparison of Anti-Biofilm Effects against Carbapenem-Resistant *Acinetobacter baumannii*: Imipenem, Colistin, Tigecycline, Rifampicin and Combinations

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Background: Multi-drug resistant (MDR) *Acinetobacter baumannii* has emerged as one of the most important nosocomial pathogens. In addition to the diverse resistance mechanisms, some *A. baumannii* strains are known to have biofilm-producing capacity, thereby decreasing antibiotic effectiveness.

Materials and Methods: This study was designed to assess biofilm-producing capacity of three different MDR *A. baumannii* strains with diverse resistance mechanisms (OXA-51, IMP-1 and VIM-2 type β-lactamases), and intended to compare the effect of each antibiotic regimen (rifampicin, colistin, imipenem, tigecycline, rifampicin-imipenem and rifampicin-colistin) on mature *A. baumannii* biofilms using *in vitro* polystyrene plate biofilm assay.

Results: Among three MDR *A. baumannii* strains, only VIM-2 strain produced strong biofilm compared to the controls (optical density, 8.04 ± 2.16 vs. 0.49 ± 0.26). Regarding VIM-2 strains, none of imipenem, colistin and rifampicin reduced biofilm formation alone at MIC of each antibiotic agent (inhibition of biofilm synthesis, less than 30%). In comparison, tigecyclin (0.76 ± 0.23), imipenem-rifampicin (1.07 ± 0.31) and colistin-rifampicin (1.47 ± 0.54) showed a significant inhibition of biofilm synthesis compared to the positive controls at 48 hours after incubation (P<0.01). Tigecycline inhibited biofilm formation even at the one fourth level of MIC (1.17 ± 0.21). Likewise, both imipenem and colistin were also effective even with the reduced concentrations when those were combined with rifampicin. Such biofilm-inhibiting effects with those antibiotic regimens sustained up to 96 hours after incubation.

Conclusion: Tigecycline, imipenem-rifampicin and colistin-rifampicin would be effective for the prevention or reduction of biofilm formation caused by *A. baumannii* strains.

Key Words: *Acinetobacter baumannii*; Biofilm; Anti-bacterial agents

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Introduction

Multidrug resistant (MDR) *Acinetobacter baumannii* has emerged as one of the most important nosocomial pathogens. Over the past several years, carbapenem-resistance rates among *A. baumannii* have markedly increased with limited therapeutic options [1]. The infections caused by MDR *A. baumannii* strains have a mortality of 25-34% associated with inappropriate antibiotic treatment [2, 3].

In addition to the diverse resistance mechanisms, some *A. baumannii* strains have biofilm-producing capacity, thereby further decreasing antibiotic effectiveness [4, 5]. Similar to the staphylococcal biofilm-associated protein (Bap), Bap has been identified in *A. baumannii* isolates [6]. Biofilms represent highly-structured communities of bacteria that are attached to a surface, and embedded in a self-produced matrix composed of extra-cellular polymeric substances [7-9]. Compared to the free-floating planktonic cells that cause acute bacterial infection, sessile cells in biofilms may lead to chronic device-related infection owing to several unique characteristics: the presence of a population that has survived unrelated to rapid multiplication, increasing resistance to antimicrobial agents, and enhanced interactions including horizontal gene transfer and co-metabolism among bacterial populations [10].

This study was designed to compare the effects of rifampicin, colistin, imipenem, tigecycline, and their combinations, on *in vitro* biofilm formation by clinical *A. baumannii* strains.

Materials and Methods

1. Bacterial strains

Three clinical carbapenem-resistant *A. baumannii* strains were included that showed different resistance mechanisms (OXA-51 with IS*Aba1*, IMP-1, and VIM-2 type β-lactamases). Acinetobacter species was initially identified with the Vitek2 system (bioMérieux, Hazelwood, MO, USA), and finally confirmed by partial *rpoB* gene sequences [11] Polymerase chain reactions (PCRs) to detect carbapenemases were performed as previously-described methods: Ambler class B carbapenemase $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2}}$, $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$, and Ambler class D OXA-type carbapenemases [12, 13]. The presence of the insertion element IS*Aba1* upstream of $bla_{\text{OXA-51-like}}$ was investigated by PCR [14].

Each *A. baumannii* strain was grown on Trypticase soy (TS) agar. The cultures were incubated at 37℃ overnight. The inoculates were prepared by touching a well-isolated agar colony with a sterile micropipette tip and then suspending the bacteria in 50 mL of TS broth by pipetting up and down several times. The bacterial suspension was adjusted to 1×10^7 CFU/mL by spectrophotometry.

2. Conditioning of polystyrene plate

To enhance biofilm formation, 600 μL of 10% bovine serum albumin (Bioworld Co., Dublin, OH, USA) was placed in polystyrene plates on a rocker at 37℃ for two hours.

3. Polystyrene microtiter plate biofilm assay

Quantification of biofilm production was carried out using a polystyrene microtiter plate assay with some modifications [4, 15]. The wells of a sterile 24-well flat-bottomed polystyrene microplates (JET Biochemicals Int Inc., Ontaria, Canada) were filled with 600 μL of overnight inoculum in TS broth before the plates were sealed with paraffin and incubated aerobically at 37℃ for 48-96 hours; the growth medium was discarded and replaced every 24 hours. To visualize the biofilms, the content of the wells were aspirated with an injector and the wells washed three times with 200 mL of sterile PBS to remove the loosely adherent cells. After drying in air, the wells were stained with 100 mL of 1% crystal violet solution for 5 minutes at room temperature. The excess stain was rinsed off by placing the plate under running tap water. Thereafter, the plates were dried at 37℃ and incubated for 30 minutes to ensure that they were dry. After the stained biofilms were resolubilized with 300 uL of ethanol-acetic acid (1:1), they were transferred to 96-well polystyrene microplates. The optical density (OD) of each well was then quantified spectrophotometically at 570 nm using a Sunrise absorbance reader (Tecan, Maennedorf, Switzerland). All tests were carried out six times (two separate experiments) for each sample. The results were expressed as the mean ± standard deviation. The cutoff value (ODc) for determining biofilm formation was defined as two times higher than the negative control value. Based on the OD values, strains were classified as the following three categories: non-biofilm producer (OD ≤ODc), weak biofilm producer (ODc<OD≤2 × ODc) or strong biofilm producer (2 × ODc <OD).

4. Comparison of mono- and combined- antimicrobial regimens on inhibition of biofilm synthesis

To elucidate the anti-biofilm effect of antibiotics against the strong biofilm-producing *A. baumanii* strain, various antibiotic regimens at each minimal inhibitory concentration (MIC) were added after 24 hours of incubation: imipenem (Choong-

Wae Pharm; Seoul, Korea), colistin sulfate (Sigma Co.; St. Louis, MO, USA), tigecycline (Wyeth Co.; Andover, MA, USA), rifampicin (Donginbiotech Co.; Seoul, Korea), imipenem-rifampicin and colistin-rifampicin. As for the antibiotics which show remarkable biofilm inhibition compared to the positive control at MIC, polystyrene microtiter plate biofilm assay was performed at MIC, half MIC and one fourth MIC simultaneously. As a combination regimen, the rifampicin concentration was kept at MIC levels with the diverse concentrations of imipenem or colistin. For the regimens with good biofilm inhibition at 48 hours after incubation, the antibiotic efficacy against biofilm production was also evaluated at 96 hours after incubation; antibiotics and growth media were replaced every 24 hours. The percentage of biofilm reduction in the presence of each antibiotic agent was compared according to the following formula: ([OD without antibiotics - OD with antibiotics] / OD without antibiotics) × 100.

5. Definition of positive control and negative control

OD values from the wells that had not been inoculated with bacteria were used as negative control. Positive control was defined as OD values from the wells where bacteria were inoculated without antibiotics.

6. Statistical analysis

All statistical analyses were performed by using SPSS version 10.0. Student's *t*-test was performed to evaluate the differences between the OD values obtained without antibiotics (positive control) and those observed in the presence of diverse antibiotic agents with different concentrations. *P*-value <0.05 was considered statistically significant.

Table 1. Polystyrene microtiter plate assay results for three carbepenem-resistant *Acinetobacter baumannii* strains

Carbapenemase	Biofilm-formation assay (OD_{570})
OXA-51[a]	0.73 ± 0.62
IMP-1[a]	0.66 ± 0.73
VIM-2[a]	8.04 ± 2.16
Negative control[b]	0.75 ± 0.35

OD, optical density; OXA, oxacillinase; IMP, imipenemase; VIM, verona imipenemase.
[a]Tests was carried out six times and the results were expressed as mean ± standard deviation.
[b]Negative control; tests was carried out 36 times and the results were expressed as mean ± standard deviation.

Table 2. Comparison of anti-biofim effects of diverse antibiotics on VIM-2 *Acinetobacter baumannii* after 48 hours of incubation

	Antibiotic concentration	OD_{570} ± SD	*P*-value[a]	Biofilm reduction (%)
Negative control[b]	-	0.75 ± 0.35	-	-
Positive control[c]	-	8.04 ± 2.16	-	-
Colistin[d]	MIC (2 mg/L)[e]	5.72 ± 0.13	0.01	28.9
Rifampicin[d]	MIC (8 mg/L)[f]	5.96 ± 1.39	0.13	25.9
Imipenem[d]	MIC (64 mg/L)[g]	7.09 ± 0.43	0.03	11.9
Tigecycline[d]	MIC (2 mg/L)[h]	0.76 ± 0.23	<0.01	90.6
	1/2 MIC (1 mg/L)	1.29 ± 0.45	<0.01	84.0
	1/4 MIC (0.5 mg/L)	1.17 ± 0.21	<0.01	85.5
Colistin-rifampicin[d]	MIC (2 mg/L)- MIC (8 mg/L)	1.47 ± 0.54	<0.01	81.8
	1/2 MIC (1 mg/L)- MIC (8 mg/L)	2.42 ± 0.31	<0.01	70.0
Imipenem-rifampicin[d]	MIC (64 mg/L)- MIC (8 mg/L)	1.07 ± 0.31	<0.01	86.7
	1/2 MIC (32 mg/L)- MIC (8 mg/L)	1.02 ± 0.15	<0.01	87.4
	1/4 MIC (16 mg/L)- MIC (8 mg/L)	1.03 ± 0.29	<0.01	87.2

[a]Comparison between positive control and each antibiotic regimen.
[b]Negative control; tests were carried out 36 times and the results were expressed as the mean ± SD.
[c]Positive control means OD values from the wells where bacteria were inoculated without antibiotics; tests were carried out 36 times and the results were expressed as the mean ± SD.
[d]Tests were carried out six times and the results were expressed as the mean ± SD.
[e]Susceptible ≤4, resistant ≥8 by British Society for Antimicrobial Chemotherapy.
[f]Susceptible ≤2, resistant ≥4 by working party report of the British Society for Antimicrobial Chemotherapy.
[g]Susceptible ≤4, resistant ≥16 by CLSI.
[h]Susceptible ≤2, resistant ≥8 by Wyeth research.
VIM, verona imipenemase; OD, optical density; SD, standard deviation; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute.

Results

According to the results of the polystyrene microtiter plate assay, only the VIM-2 type β-lactamase-producing strain among the three carbapenem-resistant *A. baumannii* strains formed strong biofilms on the polystyrene surface; the OD values ranged from 8.04 ± 2.16 (VIM-2), to 0.66 ± 0.73 (IMP-1) and 0.73 ± 0.62 (OXA-51), compared to 0.75 ± 0.35 as the negative control (Table 1).

Regarding the VIM-2 strains, imipenem, colistin, and rifampicin insufficiently reduced biofilm formation alone at MIC of each antibiotic agent; inhibition of biofilm synthesis was less than 30% (Table 2). However, tigecyclin (0.76 ± 0.23), imipenem-rifampicin (1.07 ± 0.31) and colistin-rifampicin (1.47 ± 0.54) showed significant inhibition of biofilm synthesis compared to the positive controls at 48 hours after incubation; more than 80% of the biofilm was suppressed (P<0.01). Tigecycline inhibited biofilm formation even at one fourth MIC (1.17 ± 0.21). Similarly, both imipenem and colistin were effective at reduced concentrations (one half and one fourth MIC) when combined with rifampicin.

When biofilm formation was assessed for each antibiotic regimen at 96 hours after incubation, tigecycline (0.53 ± 0.05), imipenem-rifampicin (0.60 ± 0.13) and colistin-rifampicin (0.60 ± 0.12) were still effective at the level of MIC (Table 3). The effects on biofilm-inhibition were maintained even at the one fourth MIC level of tigecycline, and both imipenem and colistin still showed excellent biofilm inhibition at the one half MIC level when combined with rifampicin.

Discussion

Multidrug resistance among *A. baumannii* has been increasing with considerable clinical concern. In addition to the high level of antimicrobial resistance, several recent studies have shown that some MDR *A. baumannii* isolates can form large amounts of biofilm [16]. *A. baumannii* strains with biofilm-forming capacity may have bee selected for under antibiotic pressure and be more likely to acquire antibiotic resistance within biofilm communities [7, 8]. Moreover, biofilms may be a persistent source of infection. This is the first study designed to compare the anti-biofilm activity of various antibiotics with regard to MDR *A. baumannii*.

According to a previous report by Wroblewska et al. [16], the majority of *A. baumannii* strains reached the highest level of biofilm formation within 24-48 h of incubation, in a time-dependent manner. Therefore, we examined the degree of biofilm formation at 48 h incubation. Rifampicin-based regimens have been shown to be effective against carbapenem-resistant

Table 3. Comparison of anti-biofim effects of diverse antibiotics on VIM-2 *Acinetobacter baumannii* after 96 hours of incubation

	Antibiotic concentration	OD_{570} ± SD	*P*-value[a]	Biofilm reduction (%)
Negative control[b]	-	0.55 ± 0.15	-	-
Positive control[c]	-	11.29 ± 0.16	-	-
Tigecycline[d]	MIC (2 mg/L)[e]	0.53 ± 0.05	<0.01	95.3
	1/2 MIC (1 mg/L)	0.80 ± 0.14	<0.01	92.9
	1/4 MIC (0.5 mg/L)	0.96 ± 0.09	<0.01	91.5
Colistin-rifampicin[d]	MIC (2 mg/L)[f] - MIC (8 mg/L)[g]	0.60 ± 0.12	<0.01	94.7
	1/2 MIC (1 mg/L)- MIC (8 mg/L)	0.85 ± 0.18	<0.01	92.5
Imipenem-rifampicin[d]	MIC (64 mg/L)[h]- MIC (8 mg/L)	0.60 ± 0.13	<0.01	94.7
	1/2 MIC (32 mg/L)- MIC (8 mg/L)	0.56 ± 0.64	<0.01	95.0
	1/4 MIC (16 mg/L)- MIC (8 mg/L)	0.62 ± 0.23	<0.01	94.5

[a]Comparison between positive control and each antibiotic regimen.
[b]Negative control; tests were carried out 18 times and the results were expressed as the mean ± SD.
[c]Positive control means OD values from the wells where bacteria were inoculated without antibiotics; tests were carried out 18 times and the results were expressed as the mean ± SD.
[d]Tests were carried out six times and the results were expressed as the mean ± standard deviation.
[e]Susceptible ≤2, resistant ≥8 by Wyeth research.
[f]Susceptible ≤4, resistant ≥8 by British Society for Antimicrobial Chemotherapy.
[g]Susceptible ≤2, resistant ≥4 by working party report of the British Society for Antimicrobial Chemotherapy.
[h]Susceptible ≤4, resistant ≥16 by CLSI.
VIM, verona imipenemase; OD, optical density; SD, standard deviation; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute.

A. baumannii infection in previous *in vivo* and clinical studies [17, 18]; however, rifampicin itself was not effective in the inhibition of biofilm formation in this study. In addition, imipenem, colistin, and rifampicin were not effective against *A. baumannii* biofilm at MIC levels. However, both imipenem-rifampicin and colistin-rifampicin combinations inhibited biofilm comparable to the negative controls. In a previous *in vivo* study reported by Song et al. [17], imipenem-rifampicin was the most effective regimen against pneumonia caused by the biofilm-forming VIM-2 strain, followed by a colistin-rifampicin combination. Tigecycline was highly active against *A. baumannii* biofilm, which effectively inhibited biofilm formation even at one-fourth MIC levels. According to the previous report by Song et al, however, tigecycline showed only bacteriostatic effects in the *in vitro* time-kill assay, and it was not effective against VIM-2 *A. baumannii* in a mouse pneumonia model [12, 17]. We assumed that tigecycline might block biofilm formation in *A. baumannii* strains apart from its antibacterial effect. Kvist et al. [19] reported that efflux pumps were highly activated in bacterial biofilms; bacteria rely on the efflux pumps to get rid of toxic substances. Tigecycline might affect the efflux pumps, quorum-sensing modulation via ribosome interaction or other biofilm-forming steps in the *A. baumannii* strains.

Previously, some have reported no correlation between biofilm formation and molecular type/carbapenemase production. However, PER-1 extended-spectrum beta-lactamase has been suggested to be related to the cell adhesion of *A. baumannii* strains [4]. According to the results of this study, there is a chance that VIM-2 carbapenemase might be related to the biofilm-forming capacity, but more VIM-2 strains need to be investigated.

In this study, we assessed antibiotic effects of various regimens on the inhibition of biofilm formation. However, the results would be different in clinical situations where large amounts of biofilm already exist.

In conclusion, tigecycline, imipenem-rifampicin and colistin-rifampicin were effective for the prevention and reduction of biofilm formation caused by *A. baumannii* strains.

Conflicts of Interest

No conflicts of interest.

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