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Association of biofilm production with colonization among clinical isolates of *Acinetobacter baumannii*

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Background/Aims: The pathogen *Acinetobacter baumannii* is increasingly causing healthcare-associated infections worldwide, particularly in intensive care units. Biofilm formation, a factor contributing to the virulence of *A. baumannii*, is associated with long-term persistence in hospital environments. The present study investigates the clinical impact of biofilm production on colonization and acquisition after patient admission.

Methods: Forty-nine *A. baumannii* isolates were obtained between August and November 2013 from Keimyung University Dongsan Medical Center, Daegu, Korea. All isolates were obtained from sputum samples of new patients infected or colonized by *A. baumannii*. The microtiter plate assay was used to determine biofilm formation.

Results: Twenty-four *A. baumannii* isolates (48%) demonstrated enhanced biofilm formation capacity than that of the standard *A. baumannii* strain (ATCC 19606). All isolates were resistant to carbapenem, 38 isolates (77%) were collected from patients in an intensive care unit, and 47 isolates (95%) were from patients who had been exposed to antibiotics in the previous month. The median duration of colonization was longer for biofilm-producing isolates than that of the biofilm non-biofilm producing isolates (18 days vs. 12 days, $p < 0.05$). Simultaneous colonization with other bacteria was more common for biofilm-producing isolates than that for the non-biofilm producing isolates. The most prevalent co-colonizing bacteria was *Staphylococcus aureus*.

Conclusions: Biofilm-producing isolates seem to colonize the respiratory tract for longer durations than the non-biofilm producing isolates. During colonization, biofilm producers promote co-colonization by other bacteria, particularly *S. aureus*. Additional research is required to determine possible links between biofilm formation and nosocomial infection.

Keywords: Acinetobacter baumannii; Biofilms; Cross infection

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INTRODUCTION

An important risk factor for nosocomial infection is prior colonization by multidrug resistant organisms. *Acinetobacter baumannii* have emerged as a major cause of hospital-acquired infections, especially for ventilator-associated pneumonia in Asia and Europe [1]. High prevalence of *A. baumannii* in ventilator-associated pneumonia and hospital-acquired pneumonia in Asian countries is a well-known problem [2]. From an epidemiological point of view, the most remarkable features of *A. baumannii* are its long-term survival in the environment

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and development of resistance to most antimicrobial agents [3].

Biofilm formation is one of the virulence factors of *A. baumannii* associated with long-term survival in a hospital environment [4]. *A. baumannii* can survive on fingertips, plastics, other environmental surfaces, and even dry surfaces. Biofilm formation contributes to this taxon's high level of resistance to desiccation and disinfection, facilitating the survival of bacteria in a hospital setting [5]. Moreover, the ability to form biofilm facilitates contact with susceptible patients, leading to outbreaks of medical device-related infections and ventilator-associated pneumonia [6,7].

Previous studies report several risk factors for acquiring *A. baumannii* after admission. These risk factors are prolonged hospitalization; prior antibiotic usage; residence in an intensive care unit (ICU); presence of foreign devices; high colonization pressure; and prolonged mechanical ventilation [8,9]. However, relatively few studies address the biofilm effect on colonization and acquisition of *A. baumannii* after admission. The aim of the present study was to determine the impact of biofilm production on colonization and acquisition of *A. baumannii*.

METHODS

Bacterial strains

A total of 49 *A. baumannii* isolates from sputum were obtained between August and November of 2013 at Keimyung University Dongsan Medical Center, Daegu, Korea. All isolates were collected from patients newly infected or colonized by *A. baumannii*. The isolates were identified using conventional biochemical methods and 16S rRNA sequencing. *A. baumannii* ATCC 19606 (ATCC, Manassas, VA, USA) was used as a positive control in the biofilm formation assay, and *Escherichia coli* DH-5α (ATCC) was used as a negative control. *A. baumannii* ATCC 19606, *E. coli* DH-5α, and the 49 clinical isolates of *A. baumannii* were maintained on Muller-Hinton agar at 35°C.

Antibiotic susceptibility testing was conducted using a VITEK II system with NH cards (bioMérieux, Marcy-l'Étoile, France), following the methodology and breakpoints defined by the Clinical and Laboratory Standards Institute.

Variables and definitions

The following patient data were collected: age, sex, date of admission, date of discharge, underlying conditions, invasive procedures, antibiotic usage in the previous 1 month, infection or colonization, hospital stay before and after isolation, therapy, and 30-day mortality. Pneumonia was diagnosed according to criteria developed by the Centers for Disease Control and Prevention (United States) [10].

We defined colonization duration as the time from the day of the first *A. baumannii*-positive culture to the first day when *A. baumannii* was not detected in culture. Persistent colonization was the sustained detection of *A. baumannii* until the final culture report. In addition, we required the final culture study to have been conducted within 1 week of the day of discharge or death. Clear-up meant that *A. baumannii* was no longer reported by any subsequent culture tests. This study was approved by the Institutional Review Board of Keimyung University Dongsan Medical Center (IRB 2013-11-040-004).

Biofilm formation assay

Biofilm formation was assayed by crystal violet staining, as described previously [11]. Fresh bacterial suspensions were prepared from overnight cultures, and each were adjusted to an optical density (OD_{600}) of 0.1. The bacterial suspensions (100 μL) were inoculated in individual wells of a 96-well plate and incubated at 35°C for 24 hours. After an overnight incubation, the plates were gently washed twice with 200 μL phosphate-buffered saline (PBS), air-dried, and stained with 0.1% crystal violet (100 μL) for 15 minutes at room temperature. Plates were gently washed twice with PBS, the stains were solubilized with 99% ethanol, and the OD_{570} of the supernatant in each well was measured using a Victor 3 microplate reader (PerkinElmer, Waltham, MA, USA). Biofilm-producing isolates were defined as those for which the optical density of solubilized crystal violet in 99% ethanol was higher than the average optical density of solubilized crystal violet for *A. baumannii* ATCC 19606. Average optical density of solubilized crystal violet for *A. baumannii* ATCC 19606 was calculated using measurements of the positive control well on each plate. All experiments were performed in triplicate. Each plate included the following controls: media alone, *A. baumannii* ATCC 19606 (positive control), and *E. coli* DH-5α

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(negative control).

Statistical analysis

Data management and statistical analyses were performed with SPSS software version 21.0 (IBM Co., Armonk, NY, USA). All data were first subjected to bivariate analysis. Categorical variables were compared using the chi-square test or Fisher exact test, and continuous variables were compared using the Mann-Whitney *U* test. Conditions contributing to colonization duration were analyzed using chi-square univariate analysis; those conditions that were significant were included in a subsequent multiple logistic regression analysis to calculate confidence intervals. All tests of significance were two-tailed. We considered tests significant when they had *p* values of 0.05 or below.

RESULTS

Biofilm mass of *A. baumannii*

The ability of *A. baumannii* ATCC 19606 to form a biofilm is established [12]. Of the 49 isolates examined, 24 (48%) exhibited enhanced biofilm formation capacity relative to a standard *A. baumannii* strain (Fig. 1).

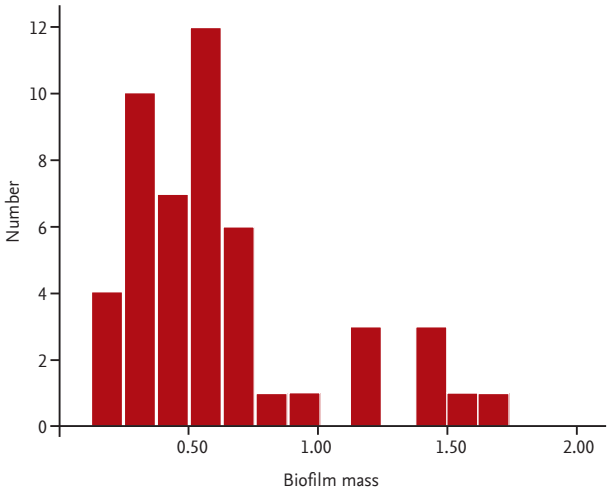

Figure 1. The biofilm mass of *Acinetobacter baumannii* clinical isolates. To measure the relative amount of the biofilm, the crystal violet stained biofilm was solubilized with ethanol for 5 minutes. Solubilized crystal violet was measured at optical density (OD_{570nm}) using a Victor 3 microplate reader (PerkinElmer). Biofilm-producing isolates were defined as those wells for which the optical density was higher than the average well optical density for *A. baumannii* ATCC 19606. The average well optical density for the standard strain of *A. baumannii* ATCC 19606 was 0.513.

Antibiotic resistance of *A. baumannii*

Antibiotic resistances were similar between the biofilm-producer group and the non-producer group. All isolates were carbapenem resistant *A. baumannii*. Thirty-eight isolates (77%) were collected from patients in the ICU and a total of 47 patients (95%) were exposed to antibiotics within the previous month. Resistance to aminoglycosides and tigecycline seemed to be higher in the biofilm non-producer group than in the biofilm producer group, with no statistical significance (Table 1).

Clinical characteristics of biofilm producer hosts and biofilm non-producer hosts

The average patient age was lower for the biofilm producer group (66.5 vs. 75, $p = 0.017$). There was no statistical difference in the underlying diseases of patients harboring biofilm producers versus those harboring biofilm non-producers. Twenty-seven patients (55%) had an endotracheal tube and 17 (34%) were on mechanical ventilation. No underlying patient disease was more common to the biofilm producer group (Table 2).

Impact of biofilm on colonization *of A. baumannii*

Thirty-seven isolates (75%) had persistently colonized their host patient's respiratory tract. The median duration of colonization was longer for biofilm-producing isolates than it was for biofilm non-producing isolates. Simultaneous colonization with other bacteria was more common for biofilm-producing isolates. The most prevalent co-colonizing bacteria were *Staphylococcus aureus*. The median timespan from admission to acquisition seemed to be shorter for the biofilm-producing group; however, this difference was not statistically significant (Table 3).

Conditions associated with colonization duration

We conducted a multivariate analysis to confirm the relationship of biofilm formation and colonization duration. In the multivariate analysis, isolates with colonization durations of more than 2 weeks are significantly associated with biofilm and ICU stays of more than 2 weeks (Table 4).

Table 1. Antimicrobial resistance rates of biofilm-producing and non-producing *Acinetobacter baumannii*

Variable	Biofilm producer (n = 24)	Biofilm non-producer (n = 25)	*p* value
Amikacin	1 (4.2)	5 (20)	0.189
Ampicillin/Sulbactam	24 (100)	23 (92)	0.490
Aztreonam	24 (100)	25 (100)	
Cefepime	24 (100)	23 (92)	0.490
Cefotaxime	24 (100)	24 (96)	1.000
Ceftazidime	24 (100)	24 (96)	1.000
Ciprofloxacin	24 (100)	24 (96)	1.000
Colistin	0	0	
Gentamicin	24 (100)	24 (96)	1.000
Imipenem	24 (100)	24 (96)	1.000
Meropenem	24 (100)	24 (96)	1.000
Minocycline	4 (16.7)	6 (24)	0.725
Piperacillin	24 (100)	24 (96)	1.000
Piperacillin/Tazobactam	24 (100)	24 (96)	1.000
Ticarcillin/Clavulanic	24 (100)	24 (96)	1.000
Tigecycline	2 (8.3)	6 (24)	0.247
TMP/SMX	24 (100)	23 (92)	0.490

Values are presented as number (%).
TMP/SMX, trimethoprim/sulfamethoxazole.

Table 2. Epidemiologic and predisposing factors for colonization with biofilm-producing or non-producing *Acinetobacter baumannii*

Variable	Biofilm producer (n = 24)	Biofilm non-producer (n = 25)	*p* value
Age, yr	66.5 (55.5–74.75)	75 (68.5–79)	0.017
Male sex	19 (79.2)	16 (64)	0.345
No underlying disease	14 (58.3)	4 (16)	0.003
Malignancy	5 (20.8)	4 (16)	0.725
Liver cirrhosis	1 (4.2)	4 (16)	0.349
Cardiovascular disease	9 (37.5)	14 (56)	0.256
Neurologic disease	9 (37.5)	7 (28)	0.551
Diabetes mellitus	5 (20.8)	5 (20)	1.000
Chronic lung disease	3 (12.5)	5 (20)	0.702
Urinary catheter	18 (75)	20 (80)	0.742
Central venous catheter	9 (37.5)	16 (64)	0.089
Parenteral nutrition	12 (50)	14 (56)	0.778
Endotracheal tube	13 (54.2)	14 (56)	1.000
Mechanical ventilator	7 (29.2)	10 (40)	0.551

Values are presented as median (interquartile range) or number (%).

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Table 3. The impact of biofilm on colonization by *Acinetobacter baumannii*

Variable	Biofilm producer (n = 24)	Biofilm non-producer (n = 25)	*p* value
Persistence	18 (75)	19 (76)	1.000
Clear-up	1 (4.2)	1 (4.0)	1.000
Duration, day	18 (8–47.75)	12 (2.5–23)	0.044
Admission to colonization, day	9 (4–22.5)	12 (7.5–15.75)	0.299
Pathogen	2 (8.3)	5 (20)	0.417
Colonization	22 (91.7)	20 (80)	
Concomitant bacteria	18 (75)	11 (44)	0.042
Staphylococcus aureus	12 (66.7)	5 (45.5)	0.438
Pseudomonas aeruginosa	5 (27.8)	2 (18.32)	0.677
Klebsiella pneumoniae	5 (27.8)	3 (27.3)	1.000

Values are presented as number (%) or median (interquartile range).

Table 4. Multivariate analysis for conditions contributing to colonization duration

Variable	Colonization duration ≥ 2 wk (n = 22)	Colonization duration < 2 wk (n = 27)	Univariate analysis		Multivariate analysis	
			Unadjusted OR (95% CI)	*p* value	Adjusted OR (95% CI)	*p* value
Biofilm	16 (72.7)	8 (29.6)	6.333 (1.814–22.107)	0.004	7.809 (1.305–46.736)	0.024
ICU stay > 2 wk	15 (83.3)	6 (30)	11.667 (2.438–55.833)	0.001	12.363 (1.927–79.310)	0.008
Endotracheal tube	15 (68.2)	12 (44.4)	2.679 (0.827–8.675)	0.149	2.080 (0.355–12.202)	0.417

Values are presented as number (%).
OR, odds ratio; CI, confidence interval; ICU, intensive care unit.

DISCUSSION

In the present study, we evaluated the impact of biofilm on host acquisition and colonization for *A. baumannii*. The biofilm-producing isolates seem to colonize the respiratory tract for longer durations than isolates not producing biofilm. During the colonization, biofilm producers also facilitate co-colonization by other bacteria, particularly *S. aureus*.

Previously, we reported higher rates of biofilm production by Korean *A. baumannii* nosocomial samples [13]. Similarly, biofilm production in the present study varies extensively among strains, and 48% of the *A. baumannii* clinical isolates exhibit a greater capacity for biofilm formation than *A. baumannii* ATCC 19606 exhibits. There are well known contributors to *A. baumannii* acquisition, such as colonization pressure, ICU admission, duration of hospitalization, and prior antibiotic use. Previous studies confirm that acquisition of multidrug resistant *A. baumannii* positively correlates with colonization pressure [8]. In addition, duration of hospitalization before admission is an important risk factor for infection by multidrug resistant *A. baumannii* [9]. Long-term care facilities are major reservoirs of multidrug resistant bacteria. Current wound management, *in situ* medical devices, and pressure ulcers are risk factors for multidrug resistant bacteria colonization in long-term care facilities [14].

In this study, biofilm-producing strains colonize the patient's respiratory tract for longer durations than biofilm non-producing strains. The longer colonization lasts, the higher the colonization pressure climbs. Heightened colonization pressure means that larger numbers of naive patients acquire multidrug resistant *A. baumannii*. Previous studies on ventilator-associated pneumonia report that airway colonization, biofilm formation, and pneumonia development have a microbial link [15,16]. Biofilm-producing strains colonize the pa-

tient's respiratory tract for longer and put the patient at high risk of developing pneumonia.

There are many reports of *A. baumannii* interacting with abiotic surfaces. However, few studies address the interaction of biotic surfaces with other bacteria, or fungi. Previous studies report that *S. aureus* and *Candida albicans* can co-exist in biofilm with synergistic effects. The biofilm mass of *S. aureus* and *C. albicans* is much higher when they grow together. Scanning electron microscope images reveal extensive adherence of *S. aureus* to hyphae of *C. albicans* [17]. In a related study, *A. baumannii* was co-cultured with *C. albicans*. This study reveals that *A. baumannii* 19606 attaches to *C. albicans* filaments, forming aggregates on the surfaces of the fungal filaments. Deletion of the *OmpA* gene results in a defect in the interaction of *C. albicans* and *A. baumannii*.

Also, *A. baumannii* interacts with the human alveolar epithelial cell. Attachment of *A. baumannii* causes human alveolar epithelial cell rounding, loss of cell projections, and their detachment from the plates [18]. The effect of biofilm on interaction with human bronchial epithelial cells is also reported. *A. baumannii* isolates carrying bla_{PER-1} show a heightened capacity for epithelial cell adherence and biofilm formation. The results show that biofilm formation correlates with epithelial cell adherence [4]. This is a possible explanation for biofilm forming multidrug resistant *A. baumannii* correlating with poor outcomes in hospital-acquired pneumonia. In addition, biofilm associated infections are more resistant to antimicrobial agents. Because of such circumstances, we use interventions such as contact precautions, environmental cleaning, active surveillance, and restrictions on administering broad-spectrum antibiotics for controlling *A. baumannii*.

This study has several limitations. We do not investigate the actual connection to infection from the colonization. Also, most of the isolates were colonization and multidrug resistant organism. We cannot evaluate the effect of biofilm on infection, clinical outcome, or antibiotic resistance. This study is retrospective, and the isolates were collected at a single center over a short duration. Further prospective studies conducted in larger patient populations involving multiple centers are needed. Biofilm-forming strains co-colonize with *S. aureus*. However, little is known about the interactions between *A. baumannii* and *S. aureus*.

These results suggest the need for further investigation of interactions between *A. baumannii* and other bacteria. Additional research is needed on possible links between colonization of biofilm-producing strains and nosocomial infections.

KEY MESSAGE

1. Biofilm production can vary extensively among the strains. Forty-eight percent of the *Acinetobacter baumannii* clinical isolates exhibited a greater capacity for biofilm formation than exhibited by *A. baumannii* ATCC 19606.
2. The median duration of colonization was longer for biofilm-producing isolates than for biofilm non-producing isolates.
3. Simultaneous colonization with other bacteria was more common in biofilm-producing isolates. The most prevalent co-colonizing bacteria were *Staphylococcus aureus*.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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