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ORIGINAL ARTICLE

Investigation of Biofilm Formation and its Association with the Molecular and Clinical Characteristics of Methicillin-resistant *Staphylococcus aureus*

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Abstract
Objectives: To investigate the biofilm-forming related factors against MRSA bloodstream isolates and evaluates their clinical features and treatment outcomes by biofilm production.
Methods: We collected 126 consecutive methicillin-resistant *Staphylococcus aureus* (MRSA) causing blood stream infections (BSIs) at 10 tertiary hospitals from 2007 to 2009. We investigated biofilm-forming ability using a microtiter plate assay, and molecular characteristics including multilocus sequence typing, staphylococcal cassette chromosome mec and accessory gene regulator types. We compared the clinical characteristics and outcomes of patients infected with biofilm-forming and non-biofilm-forming MRSA isolates.
Results: Of the 126 samples, 86 (68.3%), including 5 strong level ($OD_{570} \geq 1.0$) and 81 weak level ($0.2 \leq OD_{570} < 1.0$), had biofilm-forming capacity. Detection of fibronectinbinding protein in biofilm-forming strains was significantly higher than biofilm non-forming ones ($p = 0.001$) and three enterotoxin genes (sec-seg-sei) islands had a high frequency regardless of biofilm production. However, biofilm-forming strains were more likely to be multidrug resistant (three or more non-β-lactam antibiotics) than biofilm non-forming ones [79.2% vs. 59.2%, $p = 0.015$, odds ratio (OR) 2.629, 95% confidence interval (CI) 1.92–5.81]. Clinical features of patients with BSIs caused by biofilm-forming MRSA strains were more likely to be hospital onset [77.9% vs. 60.0%, $p = 0.024$, OR 2.434, 95% CI 1.11–5.33) and more frequently occurred in patients with use of invasive devices [85.7% vs.

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61.2%, $p = 0.002$, OR 3.879, 95% CI 1.61−8.97]. The other clinical features were compared with the clinical outcomes of the two groups and were not significant ($p > 0.05$).
Conclusion: Biofilm-forming MRSA strains showed higher frequency of *fnbB* gene than biofilm non-forming ones and more incidence rates on particular genotypes. And, their patient's features were not significantly different between two groups in this study, except for several clinical factors.

1. Introduction

Biofilms are defined as communities of bacteria encased in a self-synthesized extracellular polymeric matrix that attaches to a biotic or abiotic surface and biofilm-forming staphylococci including *Staphylococcus aureus* and *Staphylococcus epidermidis* in gram-positive bacteria have been as one of the major cause of chronic polymer-associated infection [1−3]. Infections involving biofilm-forming bacteria are extremely difficult to eradicate because biofilms impair antibiotic penetration and prevent normal immune responses [4−6]. It has been known that methicillin-resistant *S. aureus* (MRSA) cause many device-related infections and other chronic infections grow in biofilms or on these devices. Some studies have shown that it is very difficult to treat biofilm-forming staphylococcal infections with antibiotics [7−9]. Moreover, MRSA is the most common cause of nosocomial infections in intensive care units in Korea. Additionally, representative healthcare-associated MRSA strains have progressed to community-associated infections, as has been demonstrated in Korea [10]. However, the prevalence of community-associated MRSA strains in healthcare settings is increasing. Here, we studied the biofilm-forming ability of MRSA blood stream infections (BSIs) and analyzed the relationship between molecular characteristics and their clinical features for MRSA biofilm formers.

2. Materials and Methods

2.1. Bacterial strains and case definition

We collected MRSA isolates from consecutive unrelated patients in intensive care unit with MRSA BSI at 10 tertiary hospitals from 2007 to 2009. MRSA BSI was considered to be present if one or more blood cultures had positive results, and if clinical signs and course were consistent with MRSA infection. Further case definitions were defined, as previously described [11].

2.2. Identification and antimicrobial susceptibility testing

Identification and antimicrobial susceptibility testing were done using Vitek II (bioMérieux, Craponne, France) or MicroScan Pos Combo Panel Type 6 (Siemens, Munich, Germany) and confirmed by PCR for the presence of *mecA* gene. We used the following *S. aureus* strains: ATCC25923, ATCC29213, ATCC43300 (*mecA*, positive control) from the American Type Culture Collection (Manassas, VA, USA). In addition, antimicrobial susceptibility testing were performed with the disc diffusion method, if needed, according to the Clinical and Laboratory Standards Institute guidelines. *S. aureus* ATCC 29213 were used as quality control strains for MICs.

2.3. Biofilm formation assay

The biofilm formation assay in microtiter wells was performed as previously described [12]. First, overnight cultures were diluted 1:100 in brain heart infusion broth (Becton Dickinson and Co., Franklin Lakes, NJ, USA) supplemented with 1% glucose. Cell suspensions (200 μL) were transferred to individual wells of a flat-bottom 96-well polystyrene microtiter plate (Nunclon; Nunc, Roskilde, Denmark). After incubation at 35 °C for 48 hours, detached cells were gently rinsed three times with sterile water, and the bacteria that attached to the surface were stained with crystal violet, rewashed, and destained with 1 mL of ethanol-acetone (95:5, vol/vol). A total of 200 μL of the mixed solution was transferred to a 96-well microtiter plate for spectrophotometric analysis at optical density (OD) 570 nm. The absorbance was recorded by Micro-ELISA autoreader (Titertek Multiscan Plus; Labsystems, Helsinki, Finland). Each assay was performed in triplicate and the mean OD_{570} value of tested wells was applied to biofilm-forming ability. Uninoculated medium was used to determine background. The biofilm formation were divided into three categories in this study, the strains with $OD_{570} < 0.2$, $0.2 \leq OD_{570} < 1.0$, and $OD_{570} \geq 1.0$ were defined as biofilm non-formers, biofilm formers of week level, and strong level, based on the ODs in brain heart infusion broth with and without two supplements. *S. aureus* SA113 (ATCC35556) and *Staphylococcus epidermidis* RP62A (ATCC35984), well-characterized biofilm-forming strains, were purchased from American Type Culture Collection for use as positive controls.

2.4. Molecular typing

SCC*mec* types were determined by using a multiplex PCR strategy according to the method described by Oliveira and de Lancastre [13]. The *agr* type (1−4) was

assigned by PCR as previously described [14]. Multilocus sequence typing was carried out according to protocol previously described [15,16]. Data were analyzed by comparing the database at the multilocus sequence typing website (http://saureus.mlst.net), and the sequence type (ST) for each strain was determined.

2.5. Detection of virulence genes

All 126 strains were analyzed by PCR assay as previously described [12,17]. These included nine staphyloccocal enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*), three exfoliative toxin genes (*eta*, *etb*, *etd*), six adhesin genes (*icaA*, *icaD*, *cna*, *atl*, *fnbA*, *fnbB*), two surface-associated genes (*cap5HK*, *cap8HK*), and two staphylococcal regulators (*sarA*, *arlRS*).

2.6. Clinical features and outcome

Medical, laboratory, and pharmacy records were reviewed. Data from patients infected with biofilm-forming and non-forming MRSA isolates were compared. The data collected included age, sex, primary site, and onset of infection. In addition, clinical outcome was assessed for all assessable cases according to biofilm-forming capacity. The outcome measures used were crude mortality, MRSA-related death, and eradication of MRSA. Healthcare-associated risk factors included the following: presence of invasive devices (central venous catheter, urinary catheter, and other indwelling devices), prior use of antibiotics, residence in long-term care facility, prior hospitalization, prior surgery, receipt of hemodialysis, and prior MRSA colonization.

2.7. Statistical analysis

Data analysis was performed using Statistical Package for the Social Sciences (SPSS) Software version 10.0 (SPSS Inc., Chicago, IL, USA) and MedCalc Statistical Software version 10.0.1.0 (MedCalc Software Inc., Mariakerke, Belgium). Statistical significance was assessed via the Pearson χ^2 test or the Fisher exact test for categorical variables and the Student t test or the Mann−Whitney U test for continuous variables. Logistic regression analysis was used for multivariate analysis. Variables that achieved a probability of <0.1 in univariate analyses were considered for inclusion in logistic regression models.

3. Results

3.1. Antimicrobial susceptibility and biofilm formation

A total of 126 MRSA isolates from blood cultures were analyzed; 86 strains (68.3%) had biofilm-forming ability with five strong and 81 weak level and the other strains (40/126, 31.7%) did not form biofilms (Figure 1). All isolates were resistant to oxacillin and penicillin. Biofilm-forming isolates were less frequently resistant to oxacillin and penicillin (14.0% vs. 42.5%; $p = 0.005$), and they were more likely to be multidrug resistant (three or more non-β lactam antibiotics) than biofilm nonforming ones (85.0% vs. 57.5%; $p < 0.001$; Table 1).

3.2. Comparison of genotypic characteristics

The genetic characteristics of 126 MRSA strains were differed between biofilm-forming and nonforming isolates (Table 2). Most biofilm-forming (55 isolates; 64.0%) and biofilm-nonforming (20 isolates; 50.0%) isolates were of *agr* group II, with no difference in the distribution of the *agr* group. The most common SCC*mec* type was II in both biofilm-forming and nonforming isolates, and the next most frequent type was SCC*mec* type IV ($p = 0.055$). Biofilm-forming isolates were more likely to contain ST5 (69.8% vs. 52.5%; $p = 0.060$), ST239 (8.1% vs. 2.5%; $p = 0.434$) and significantly less likely to contain ST72 (18.6% vs. 42.5%; $p = 0.005$) than biofilm nonforming isolates.

3.3. Determination of virulence-associated genes

Table 3 presents the distribution of virulence-associated genes (adhesin-encoding, toxin-encoding, surface-associated, and gene regulators). With a range over 90%, most of the isolates had similar distribution of adhesion genes (*icaA*, *icaD*, *cna*, *atl*, and *fnbA*), toxin genes (SEs, *hla*, and *hlb*), and staphylococcal regulators (*sarA* and *arlRS*) between biofilm-forming and nonforming isolates and both isolates showed low frequency in detection of *eta*, *etb*, *etd*, PVL, and *cap8HK*. The biofilm-forming isolates were significantly more detected in distribution of *fnbB* than biofilm-nonforming isolates (74.4% vs. 45.0%; $p = 0.001$; odds ratio 3.556; 95% confidence interval 1.615−7.827).

3.4. Clinical features of patients by biofilm-forming ability

Epidemiological and clinical data were available for 126 patients and were included in the analysis of factors associated with biofilm formation. Patient characteristics are shown in Table 4. Biofilm-forming isolates were more frequently detected in nosocomial infection (77.9%) than biofilm-nonforming isolates (60%). Among 86 nosocomial infection, biofilm-forming isolates were involved in 67 cases (77.9%), which was higher than community onset cases (22.1%). There were no significant differences in sites of infection. CRI and primary infection were two most common origins of MRSA infection regardless of biofilm-forming ability. As shown in Table 4, there were no significant differences between clinical outcomes of biofilm-forming and nonforming cases for crude mortality and MRSA-related death, in univariate analysis shown in Table 5, significant risk factors associated with healthcare-associated biofilm-forming isolates were the presence of invasive devices (central venous catheter, urinary catheter, and

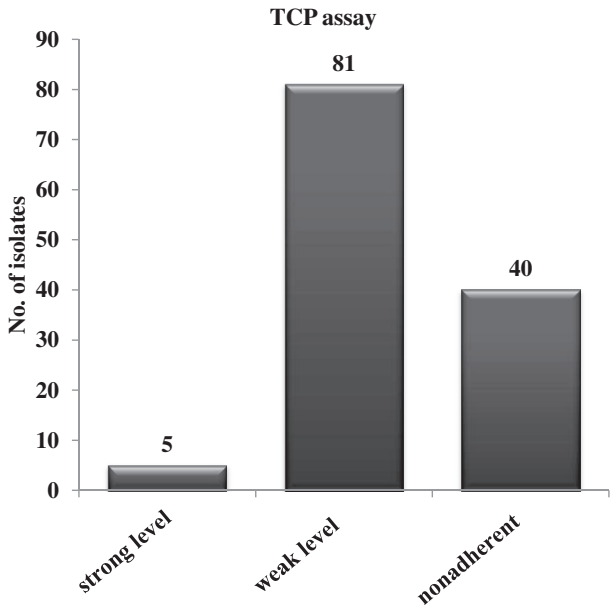

Figure 1. Biofilm-forming capacity of 126 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates by tissue culture plate (TCP) assay.

other indwelling devices) and prior hospitalization ($p < 0.05$). Odds ratios (95% confidence interval) for the variable selected in multivariate analysis were: presence of invasive devices, 3.87 (1.60−8.96); and prior hospitalization, 0.40 (0.18−0.88).

4. Discussion

The ability to form biofilm is a trait that is closely associated with bacterial persistence and virulence, and many persistent and chronic bacterial infections are now believed to be linked to the formation of biofilms [18]. In this study, we analyzed the presence of various virulence genes and adherent proteins against MRSAs causing BSI. The high proportion (about 80−95%) of MRSA isolates in our study could make no significant difference for virulence genes according to biofilm-forming capacity, except for *fnbB* gene. We found the *fnbB* gene was to be more frequent among biofilm-producing MRSA strains of ST5 (34/86 isolates) and biofilm non-producing MRSA strains of ST72 (16/40 isolates) respectively ($p = 0.005$)(data not shown). ST5 strains have been known as frequent genotype in hospital and ST72 in community environment, especially in Asia [11, 19, 20]. In our results, MRSAs of ST5 and ST72 genotypes showed a frequency of *fnbB* for either biofilm formers or non-formers. This means that the presence of *fnbB* gene may be correlated with the biofilm-forming ability and MRSA trait from a

Table 1. Comparison of antimicrobial susceptibilities between biofilm-forming and nonforming isolates

	Antibiotic resistance profiles	No. of isolates	%
Biofilm formers (86)	OX-P	12/86	13.9
	OX-P-CLI	4/86	4.7
	OX-P-TET	1/86	1.2
	OX-P-CIP-CLI	7/86	8.1
	OX-P-CLI-TET	1/86	1.2
	OX-P-CIP-TET	1/86	1.2
	OX-P-CIP-CLI-SXT	1/86	1.2
	OX-P-CIP-CLI-TET	57/86	66.2
	OX-P-CIP-CLI-TET-SXT	1/86	1.2
Biofilm non-formers (40)	OX-P	17/40	42.5
	OX-P-CIP	1/40	2.5
	OX-P-TET	1/40	2.5
	OX-P-CIP-CLI	3/40	7.5
	OX-P-CIP-CLI-TET	17/40	42.5
	OX-P-CIP-CLI-TET-SXT	1/40	2.5

CIP = ciprofloxacin; CLI = clindamycin; OX = oxacillin; P = penicillin; SXT = trimethoprim-sulfamethoxazole; TET = tetracycline.

Table 2. Genotypic distribution between biofilm-forming and nonforming isolates

Characteristic	No. (%) of MRSA isolates: Biofilm formers ($n = 86$)	Biofilm non-formers ($n = 40$)	p*
agr group			
I	25 (29.1)	18 (45.0)	0.357
II	55 (64.0)	20 (50.0)	
III	3 (3.5)	1 (2.5)	
SCC*mec* type			
I	0 (0.0)	0 (0.0)	0.055
II	60 (69.8)	20 (50.0)	
III	6 (7.0)	1 (2.5)	
IV	18 (20.9)	18 (45.0)	
MLST			
ST1	2 (2.3)	1 (2.5)	
ST5	60 (69.8)	21 (52.5)	0.060
ST8	1 (1.2)	0 (0.0)	
ST72	16 (18.6)	17 (42.5)	0.005
ST239	7 (8.1)	1 (2.5)	0.434

*The p values comparing the values for the two groups were determined using a two-sample test for binomial proportions. MLST = multilocus sequence typing; MRSA = methicillin-resistant *Staphylococcus aureus*.

certain lineage. This presumption could be accordant with a previous study [21], suggesting that FnbB-mediated biofilm development is a common MRSA trait from clonal complex (CC) 8, CC22, and CC45 lineages. However, studies of the relationship of specific STs and biofilm formation require further investigation.

Epidemiologically biofilm-forming MRSA infection was highly associated with nosocomial infection in this study. Hospital environments may be more suitable for biofilm formation. Various healthcare-associated risk factors are suggested to affect biofilm formation preferable environments to a greater or lesser extent. Our results showed that significant risk factors were the

Table 3. Distribution of virulence-associated genes by functional category

Functional category	No. of isolates by biofilm formation (%); Positive, $n = 86$	Negative, $n = 40$	p*
Adhesin-encoding genes			
icaA	83 (96.5)	38 (95.0)	0.652
icaD	86 (100)	40 (100)	
cna	80 (93.0)	36 (90.0)	0.724
atl	86 (100)	40 (100)	
fnbA	83 (96.5)	39 (97.5)	1.000
fnbB	64 (74.4)	18 (45.0)	0.001
Toxin-encoding genes			
SEs	86 (100)	40 (100)	
eta, etb, etd	1 (1.2)	0 (0.0)	1.000
tst	78 (90.7)	34 (39.5)	0.370
PVL	2 (2.3)	0 (0.0)	1.000
hla	83 (96.5)	38 (95.0)	0.652
hlb	86 (100)	39 (97.5)	0.317
Surface-associated genes			
cap5HK	75 (87.2)	35 (87.5)	0.964
cap8HK	9 (10.5)	3 (7.5)	0.751
Staphylococcal regulators			
sarA	86 (100)	40 (100)	
arlRS	80 (93.0)	39 (97.5)	0.430

The percentages refer to the percentage of patients within each *Staphylococcus aureus* subset with the indicated virulence gene.
*The p values comparing the values for the two groups were determined using a two-sided Fisher's exact test ($p < 0.05$). As determined by the Fisher exact test; all p values shown in this table (referring to a comparison of values for biofilm-forming and non-forming isolates) are statistically significant with a false discovery rate of < 20%.

Table 4. Summary of clinical characteristics

Characteristics	Biofilm formers *n* (%)	Bioflim non-formers *n* (%)	*p*	OR (CI_{95})
Age, years			0.203	0.985 (0.964−1.008)
Mean ± SD	63.8 ± 12.5	56.7 ± 14.7		
Median (range)	69 (1−98)	65 (1−80)		
Male:female	56:30	26:14	0.990	
MRSA isolates				
Onset of infection				
Nosocomial	67 (77.9)	24 (60.0)	0.024	2.434 (1.111−5.331)
Community onset	19 (22.1)	16 (40.0)		
Site of infection				
Endocarditis	2 (2.3)	0 (0)	0.255	
Lung infection	7 (8.1)	5 (12.5)	0.407	
SSTI/Bone	8 (9.3)	3 (7.5)	0.857	
CRI	31 (36.0)	21 (52.5)	0.466	
Other sites	3 (3.5)	0 (0)	0.281	
Primary	33 (38.4)	14 (35.0)	0.629	
Clinical outcome				
Crude mortality	29 (37.7)	16 (40.0)	0.423	
MRSA-related death	16 (18.6)	6 (15.0)	0.203	
Eradication of MRSA			0.824	
Success	56 (72.7)	26 (65.0)		
Failure	19 (22.1)	10 (25.0)		

CI_{95} = 95% confidence interval; CRI = catheter-related infection; MRSA = methicillin-resistant *Staphylococcus aureus*; OR = odds ratio; SD = standard deviation; SSTI, staphylococcal skin and tissue infection.

presence of invasive devices and prior hospitalization. Even though not significant statistically, proportions of patients with prior antibiotic use and prior MRSA colonization were higher in biofilm-forming isolates than nonforming isolates. However, among the community onset cases, nearly half of the isolates revealed biofilm-forming activities. This suggests that biofilm formation may be troublesome in community-associated MRSA as well as healthcare-associated MRSA.

It is widely known that biofilm might play a role in the pathogenesis of device-associated MRSA infections. Particularly, the presence of biofilms on intravascular catheters and their role in catheter-related BSI (CRBSI) is well accepted [22]. We defined CRBSI according to the Infectious Diseases Society of America guidelines [23,24], but the diagnosis of CRBSI remains a major challenge. Although proven CRBSI cases in patients with biofilm-forming isolates were lower than primary BSI in this study, biofilm-formation was significantly associated with the presence of invasive devices, which suggests that invasive devices may be the hidden focus of MRSA BSI. However, this causal relation was not proven and further studies would be necessary to investigate this possibility.

Biofilm infections are important clinically because bacteria in biofilms exhibit recalcitrance to antimicrobial compounds and persistence in spite of sustained host defenses [25]. Biofilm infection represents a reservoir of dissemination of bacterial infection to other sites in the human body [26]. During infection, attachment is a crucial part of the colonization on host tissues or on indwelling medical devices, whereas detachment

Table 5. Healthcare-associated risk factors associated with biofilm-forming capacity

Healthcare-associated risk factors	Biofilm formers (%)	Biofilm non-formers (%)	*p*	Odds ratio (95% confidence interval)
Presence of invasive device	85.7	61.2	0.002	3.87 (1.60−8.96)
Prior antibiotic use	71.6	62.5	0.535	1.37 (0.49−3.89)
Residence in LTCF	12.5	10.0	0.704	1.30 (0.33−5.12)
Prior hospitalization	60.2	40.0	0.024	0.40 (0.18−0.88)
Prior surgery	36.4	17.5	0.121	0.47 (0.17−1.22)
Receipt of hemodialysis	26.1	30.0	0.278	1.65 (0.66−4.09)
Prior MRSA colonization	33.8	15.0	0.295	0.61 (0.24−1.54)

LTCF = long-term care facility.

is a prerequisite for the dissemination of an infection. Fux et al [20] reported that the detachment of multicellular clumps may explain the high rate of symptomatic metastatic infections seen with *S. aureus*. They also revealed that nonattached aggregates of bacteria retain the antibiotic resistance seen in biofilms. Collectively, biofilm formation can lead to intractable infection and worse outcome.

However, significant differences in outcomes between biofilm-forming and nonforming cases were not observed. There are some possible explanations. First, we targeted MRSA isolates that already exhibited a high level of resistance in a significant portion, which may reduce the effects of enhanced antimicrobial resistance due to biofilms. Clinical outcomes are very complex and comprehensive products of various factors including not only bacterial factors but also host factors. Therefore, it is difficult to demonstrate the independent effects of biofilm. In particular, we included patients in intensive care units with MRSA BSI at tertiary hospitals, who generally have very severe status and showed high mortality. By contrast, evaluation of the duration of bacteremia and symptoms would produce different results, as previous studies revealed the association of biofilms and persistent infection [1,25,27], but the information was not submitted.

In summary, most MRSA isolates related to BSI produced biofilms and their genotypic characteristics have a tendency to having prevalence in some STs and specific genes. Therefore, the patients having biofilm-forming MRSAs seem to be associated with prior use of a medical device and prior hospitalization. These molecular and epidemiological analyses for biofilm-producing MRSAs could be given as basic information for patients who cannot be treated, and may be helpful in determining the possibility of biofilm-related *S. aureus* infections.

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