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Clinical and genetic risk factors for biofilm-forming *Staphylococcus aureus*

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Running Head: Genetic and Clinical Risks of MRSA Biofilms

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ABSTRACT

Background. Molecular and clinical factors associated with biofilm-forming methicillin-resistant *Staphylococcus aureus* (MRSA) are incompletely understood.

Methods. Biofilm production was quantified in 182 MRSA isolates from clinical culture sites (2004-2013). Microbiologic toxins, pigmentation, and genotypes were evaluated, and patient demographics were collected. Logistic regression was used to quantify the effect of strong biofilm production (versus weak) on clinical outcomes and independent predictors of strong biofilm.

Results. Of isolates evaluated, 25.8% (47/182) produced strong biofilm, and 40.7% (74/182) produced weak biofilm. Strong biofilm-producing isolates were more likely to be from MLST clonal complex 8 (34.0% vs. 14.9%; P=0.01), but less likely to be from MLST CC5 (48.9% vs. 73.0%, P=0.007). Predictors for strong biofilm were *spa* type t008 (aOR 4.54 95%CI 1.21-17.1), and receiving chemotherapy or immunosuppressants in the previous 90 days (aOR 33.6; 95%CI 1.68-673). Conversely, patients with high serum creatinine (aOR 0.33; 95%CI 0.15-0.72) or who previously received vancomycin (aOR 0.03; 95%CI 0.002-0.39) were less likely to harbor strong biofilm-producing MRSA. Beta-toxin producing isolates (aOR 0.31; 95%CI 0.11-0.89) and isolates with *spa* type t895 (aOR 0.02 95%CI <0.001-0.47) were less likely to produce strong biofilm. Patient outcomes also varied between the two groups. Specifically, patients with strong biofilm-forming MRSA were significantly more likely to be readmitted within 90 days (aOR 5.43; 95%CI 1.69-17.4), but tended to have decreased 90-day mortality (aOR 0.36; 95%CI 0.12-1.06).

Conclusions: Patients that harbored t008 and received immunosuppressants were more likely to have a strong biofilm-producing MRSA. Clinically, patients with strong biofilm-forming MRSA were less likely to die at 90 days, but five times more likely to be re-admitted.

BACKGROUND

Biofilms are critical for the pathogenicity of most bacteria, including *Staphylococcus.* As a result, *S. aureus* infections can develop into chronic, difficult to treat infections that require long durations of antimicrobial therapy and surgical intervention. Based on previous reports and various assays used, 43 to 88% of clinical *S. aureus* isolates can form biofilms.(1-4) Biofilm in *S. aureus* has been associated with several regulatory and virulence factors such as accessory gene regulator (*agr*) downregulation and heteroresistant vancomycin intermediate susceptibility.(5) Genotypic variation among strains may also affect biofilm production, but these relationships haven't been consistently reported.(6, 7)

Methicillin-resistant *S. aureus* (MRSA) causes significant morbidity and mortality. Risk factors for infection with MRSA are clearly defined; however, little is known about the molecular and clinical risk factors for biofilm-producing MRSA.(8-11) Defining these risk factors and understanding the clinical outcomes associated with biofilm-producing MRSA can provide critical and timely insight into the prevention and treatment of these serious infections. Further, understanding the phenotypic and genetic characteristics associated with biofilms in MRSA may enable the development of biofilm detection methods in clinical microbiology laboratories and identify therapeutic targets. Therefore, the objectives of this study were to quantify clinical outcomes among adult patients with strong biofilm-producing MRSA (OD ≥ 2.0) or weak biofilm-producing MRSA (OD ≤ 1.0) and to identify clinical and molecular independent predictors of strong biofilm-producing MRSA.

MATERIALS AND METHODS

Study design, population, and bacterial isolates. A retrospective cohort study was conducted among a sample of inpatient and outpatients with MRSA cultures from any culture site at the Providence Veterans Affairs Medical Center (PVAMC), a 119-bed federal hospital from May 2004

to October 2013. Nares swabs collected for infection control surveillance purposes were excluded. Duplicate isolates with the same multi-locus sequence typing (MLST) clonal complexes (CC) and collected from the same date or admission were excluded. Each isolate included was treated as an independent event, and therefore patients may have been included in the study more than once. This study was approved by the Institutional Review Board and the Research and Development Committee of the PVAMC.

Microbiological (phenotypic and genotypic) data.

Biofilm formation assay. Biofilm formation was determined using a modified Christensen method as previously described by our group.(12-16) *Staphylococcus epidermidis* ATCC 35984 and methicillin-susceptible *Staphylococcus aureus* ATCC 35556 were used as positive controls. The isogenic accumulation-negative mutant of ATCC 35984, M7, was used as a negative control.(17-19) After incubation, planktonic bacteria were removed by rinsing each well three times with sterile Millipore water. Plates were dried overnight then stained with 0.1% crystal violet for 15 minutes. Adherent stain was resolubilized with 33% glacial acetic acid for one hour before measuring optical density (OD) at 570 nm on a spectrophotometer (ELX800, Biotek, Winooski, VT). To obtain the final OD values the OD of wells containing tryptic soy broth (TSB) with 1.0% dextrose only (media control) were subtracted from wells containing isolates to remove background readings. Mean OD was calculated for each isolate, using at least four replicates.(17, 20) We used the degree of biofilm production; strong (OD ≥ 2.0), moderate (1.0 < OD < 2.0) and weak (OD ≤ 1.0) as previously described.(21) For this study, we excluded moderate biofilm-producing isolates.

Alpha- and Beta-toxin production. Qualitative alpha-toxin production, indicated by clear zones of hemolysis, was evaluated for each strain on Mueller-Hinton agar with 5% sheep blood after

24h incubation at 37°C.(22) Plates were then refrigerated at 4°C for 24h to evaluate beta-toxin production, indicated by green-brown hemolysis.

Determination of Agr operon function. Function of the *agr* operon was measured qualitatively by delta-toxin production.(22, 23) Delta-toxin expression was determined by streaking the MRSA test isolates adjacent to a Beta Lysin Disk (Remel, Lenexa, KS) on tryptic soy agar with 5% sheep blood and incubated at 37°C for 24 hours. The presence of synergistic hemolysis between the streak and Beta Lysin Disk indicated the production of delta-hemolysis, therefore a functional *agr* locus.(22, 23) The dysfunction of *agr* was defined as the absence of delta-hemolysis within the beta-toxin zone, as evidenced by the lack of synergistic hemolysis.(23) Reference strains RN4420 and RN6607 were used as negative and positive controls for delta-toxin, respectively.

Heterogeneous vancomycin intermediate Staphylococcus aureus (hVISA) presence. Screening for hVISA was conducted using E-test glycopeptide resistance detection (GRD) strips (bioMérieux, Durham, NC).(24) Testing was conducted according to manufacturer's instructions using a standard 0.5 McFarland bacterial suspension on Mueller-Hinton agar with 5% sheep blood (BD, Sparks, MD). The results were read at 24 and 48 hours after incubation. Standard vancomycin Etests were also conducted according to manufacturer's instructions, on Mueller-Hinton agar for 24 hours. Heteroresistance was defined as a vancomycin or teicoplanin MIC of ≥ 8 µg/mL on the GRD Etest plus a standard vancomycin MIC < 4 µg/mL. Quality control of susceptibility testing was performed with reference strain ATCC700698 (Mu3, hVISA).

Pigmentation. Golden pigmentation was evaluated after overnight growth on tryptic soy agar at 37°C.(25, 26) Each strain was compared to a reference white strain of *S. epidermidis* ATCC35984 and categorized as pigmented or non-pigmented. *S. aureus* ATCC35556 served as a pigmented

control. A selection of 60 strains were categorized independently by a second reviewer, with 98.3% agreement.

Genotyping. Staphylococcal protein A (*spa*) genotype was determined by PCR as previously described, with 1095F and 1517R primers.(27) Gene sequences were determined using Sanger sequencing with the forward primer only unless reverse primer was necessary for sequence clarification. *Spa* type was mapped to common MLST CC using the Ridom spa server (Spaserver.ridom.de). *Spa* types not matched to a clonal complex in the Ridom spa server were matched by literature search.

Patient data. Data was collected through a chart review of electronic medical records and included diagnoses and procedures, clinical measurements, microbiology data, patient demographics, health-care exposure within 90 days of index culture (hospitalization >72 hours and surgical procedures), receipt of antimicrobials or medications that may influence biofilm formation in the previous 90 days (i.e. gastric acid suppressants [proton-pump inhibitors or H_2-blockers], chronic corticosteroid use, non-steroidal anti-inflammatory [NSAID], HMG-CoA reductase inhibitors [statins])(28-32), presence of prosthetic/foreign devices (i.e. orthopedic, cardiovascular, urinary Foley, intravenous catheters), and infection/colonization history in the previous year.

Clinical outcome definitions. Clinical outcomes of interest were all-cause mortality, admission among outpatient or re-admission among inpatients, MRSA infection and MRSA-related admission among outpatients or re-admission among inpatients. As the risk period for poor outcomes in these patients is not known, we evaluated outcomes at follow-up periods of 30 and 90 days.

The index date was defined as the collection date of the MRSA isolate tested for biofilm production (index culture). MRSA infection was confirmed from microbiology data, and diagnosis of infection in the medical record. Readmission was defined as admission for any reason after the discharge date of the index culture. For index isolates collected in the outpatient setting, admission was defined as admission for any reason after the index date.

Statistical analysis. Between-group differences were assessed using X^2 or Fisher exact tests for categorical variables and the T-test or Wilcoxon rank sum test for continuous variables. Logistic regression models were used to quantify the effect of strong biofilm on each clinical outcome, while controlling for confounders of the exposure-outcome relationship.(33) In multivariable modeling, a manual, non-computer-generated backward elimination approach was implemented. Logistic regression was also used to identify independent predictors associated with strong MRSA biofilm production.(33) All baseline variables were evaluated as potential confounders in the clinical outcome models, and as independent predictors of biofilms in the predictive model. Crude and adjusted odds ratios (OR) and respective 95% confidence intervals (CI) are presented. All statistical tests were conducted using SAS, version 9.2 (SAS Institute, Cary, NC), with a two-tailed α value of 0.05 required for statistical significance.

RESULTS

Isolate and clinical characteristics. In total, 121 MRSA isolates were included for biofilm production, 38.8% (47/121) produced strong biofilms (OD ≥ 2.0) and 61.2% (74/121) produced weak biofilms (OD ≤ 1.0). Race was significantly different between the groups, with the strong biofilm group having a higher number of whites (93.6% vs. 79.7%; P=0.04). There was no difference between the groups in age, gender or BMI. Serum creatinine and creatinine clearance was significantly different between the two groups. Median (Q1-Q3) serum creatinine was 0.9 (0.8 - 1.1mg/dl) for the strong biofilm group vs 1.3 (0.9 - 2.2 mg/dL) for the weak biofilm group (P=0.001). The median (Q1 – Q3) creatinine clearance was 92.6 (range 67.6-117.6 mL/min) in the strong biofilm group vs 58.4 (31.7 – 89.2 mL/min) for the weak biofilm group (P=0.001). Significantly lower number of patients in the strong biofilm group had chronic renal failure (12.8% vs 31.1%; P=0.02). There was no difference between the groups in Charlson comorbidity index or other comorbidities such as diabetes, cardiovascular disease, liver disease, malignancies and anemia. The groups did not differ in IV drug use, alcohol abuse or smoking. The presence of foreign material/device was lower in patients with a strong biofilm-producing isolate (25.5% vs 50.0%; P=0.01). Significantly lower number of patients in the strong biofilm group were hospitalized for two or more days in the previous 90 days (27.7% vs 52.7%; P=0.007). Overall antimicrobial use in the previous 90 days from culture was not significantly different between the groups but use of vancomycin was significant with 27.0% of patients receiving vancomycin in the weak biofilm group whereas only 2.1% in the strong biofilm group (P=0.001). There were fewer patients on hemodialysis in the strong biofilm-producing group (0% vs. 13.5%; P=0.006). Patients in the strong biofilm group had a lower number of bacteremias (4.3% vs 17.6%; P=0.03) and pneumonias (10.6% vs 25.7%; P=0.04) in the year prior to the index culture. Patients in the strong biofilm group tended to present in the outpatient setting at the index culture (51.1% vs 32.4%; P=0.04). **(Table 1)**.

Alpha-toxin was produced by 79.3% (n=96) of the isolates overall (74.5% strong biofilm vs 82.4%weak biofilm, p=0.29). Beta-toxin production was less common, with 69.4% (n=84) of isolates (59.6% strong biofilm vs 75.7% weak biofilm, p=0.06). Presence of hVISA was rare among strong biofilm and weak biofilm-producing isolates (8.5% vs 4.4%; P=0.44). The proportion of isolates with *agr* dysfunction (61.7% vs 43.2%; P=0.05) and pigmentation (76.6% vs 54.1%; P=0.01) were significantly higher in the strong biofilm group. The distribution of vancomycin MIC was similar among both groups. MRSA isolates represented seven MLST CC, the most common were CC5 (63.6%) and CC8 (22.3%). Strong biofilm-producing isolates had significantly lower MLST CC5 (48.9% vs 73.0%; P=0.007) and significantly higher CC8 (34.0% vs 14.9%; P=0.01). There were 24 different *spa* types identified among the isolates. Of those *spa* types, the most common were t002 (32.2%), t895 (15.7%), t008 (14.9%), and t1094 (5.8%). Strong biofilm-producing isolates contained significantly more *spa* type t008 (25.5% vs 8.1%; P=0.01) and less t895 (2.1% vs 24.3%; P=0.001). **(Table 2)**.

Clinical Outcomes and Independent Predictors. After controlling for potential confounders, patients with strong biofilm-producing MRSA were more than five times as likely to be (re)-admitted within 90 days of discharge (adjusted OR 5.43; 95% CI 1.69-17.4). The strong biofilm group was 64% less likely to die within 90 days (adjusted OR 0.36; 95% CI 0.12-1.06), but this was not statistically significant. There was no difference in 30 day mortality, 30 day (re)-admission, MRSA reinfection at 30 or 90 days, or MRSA related (re)-admission at 30 or 90 days among patients with strong or weak biofilm-producing MRSA. (**Table 3)**

Patients who were on chemotherapy and/ or used immunosuppressants within 90 days of index culture had a 33.6 times higher odds for strong biofilm-producing MRSA isolate (adjusted OR 33.6; 95% CI 1.68-673). Patients harboring isolates from t008 (adjusted OR 4.54; 95% CI 1.21-17.1) also had increased risk of a strong biofilm-producing MRSA. Further, patients with isolates

from t895 (adjusted OR 0.02; 95% CI <0.001-0.47) or that produced beta-toxin were less likely to produce strong biofilm (adjusted OR 0.31; 95%CI 0.11-0.89). Patients who had increased serum creatinine (adjusted OR 0.33; 95% CI 0.15-0.72) or who received vancomycin in the previous 90 days (adjusted OR 0.03; 95%CI 0.002-0.39) were less likely to produce strong biofilm. **(Table 4)**.

DISCUSSION

This study demonstrated that strong biofilm formation among clinical MRSA isolates was associated with increased readmission at 90 days and a trend toward decreased 90-day mortality. Strong biofilm formation was also associated with MRSA lineage, *agr* dysfunction, pigmentation, and several patient factors including serum creatinine, race, and immunosuppressants.

Biofilm formation has been previously associated with patient mortality. A previous study demonstrated increased mortality with biofilm-forming isolates, but the attributable mortality was low.(3) Similar to our study, included patients were primarily male, and members of military services (however they were younger than our Veterans), but whereas our study was only in MRSA isolates, this study included multiple types of bacterial cultures and found a five-fold increased association of MRSA among the biofilm-positive group (OR 5.09, 95% CI 1.12-23.1). Overall mortality with initial infection was 16% versus 5% in biofilm versus non-biofilm group (p=0.01), with an attributable mortality of 7%.(3) Unfortunately, it is difficult to tell how many of these are due to biofilm-forming versus non-biofilm forming MRSA in the study, as opposed to other bacterial types.

The majority of MRSA isolates in our study represented CC5, typically referred to as hospital-associated strains and CC8 historically of community origin. In the multivariate analyses, there was no association between clonal complex and biofilm formation, which has been found in other studies.(34-36) This may be due to limited number of isolates, or the clinical source of the isolates used, which may play a role in their biofilm formation. However, in univariate analyses, weak biofilm isolates had more CC5, which is traditionally hospital-associated, as well as more previous hospitalization within the previous 90 days, dialysis, bacteremia and pneumonia within the previous year, and treatment with vancomycin. Although not statistically significant, weak biofilm isolates had more antimicrobial use in the prior 90 days and more infections in the previous year

in all categories. This may represent a higher severity of illness, and may help to explain the increased mortality seen at 90 days. In contrast, CC8, the traditionally community-acquired clone, has been previously associated with strong biofilm production, as well as community-acquired skin infections and colonization.(34, 37) These types of infections and colonization may be associated with lower mortality, as seen in our study. The most common *spa* types were t002, t008, t895, and t1094. Though t002 and t895 are related to CC5, t002 was not related to biofilm formation. We found that isolates from *spa* type t008 were predictive for the strong biofilm phenotype, while t895 was significantly higher in weak biofilm-producing isolates. At least for this subset of isolates, *spa* type served as a better predictor of biofilm formation than MLST CC, potentially due to the greater degree of resolution in *spa* typing. This finding is consistent with previous studies evaluating genotypically different clones of MRSA in the production of biofilm.(6, 7)

Previously published data suggest that *agr* dysfunction is associated with biofilm formation in *S. aureus*.(5, 38-40) This is in line with our own data, which demonstrated *agr* dysfunction was present in 61.7% of strong biofilm formers versus 43.2% of weak biofilm formers. Some data demonstrate conflicting results with regard to *agr* function and biofilm, depending whether the biofilm is formed in vivo or in vitro.(41) In vitro biofilm formation may yield a different relationship with *agr* than in vivo biofilms, since there is no host response-relationship. It is suggested that the host response and *agr*-dependent virulence factors secreted in vivo regulate biofilm formation.(41) Previous studies have also suggested that *agr* dysfunction is associated with hVISA development, however because our overall numbers of hVISA were low, we could not confirm this finding.(22, 42) Beta-toxin was associated with weak biofilm formation, and was a negative predictor for strong biofilm in the logistic regression model (adjusted OR 0.31; 95%CI 0.11-0.89). Although there is limited data on the connection between beta-toxin and biofilm formation, in previous studies, beta-toxin was associated with skin colonization, and colonization

was associated with a low biofilm phenotype, consistent with our findings.(43, 44) Alpha-toxin has also been associated with biofilm formation,(45, 46) however, we did not quantify how much alpha-toxin these isolates produce in this study, which may have correlated better to biofilm formation than a dichotomous presence or absence of alpha-toxin. Overall, these findings underscore the need for additional studies to better describe the mechanisms responsible for the presence of biofilms.

This study had several limitations. A limited sample size may have impaired the ability to find associations between biofilm production and covariates previously noted to play a role in biofilms. Of course, we cannot guarantee that in vitro biofilm formation equates to clinical biofilm formation in an infection. Due to the retrospective design of this study, not all variables or potential confounders may have been included in the analysis of clinical factors, and we are reliant on the accuracy of data as entered into the patient electronic medical record. To minimize selection bias, the investigator collecting clinical data was blinded to the biofilm status of each isolate. Biofilm formation is determined using a standard assay.(14, 15, 18, 19, 47) Additionally, we utilize a negative control isolate to ensure comparability between results. By removing the moderate biofilm category, we may have limited our power in the number of isolates, but the isolates had the most different biofilm classifications to see differences in the predictors and outcomes.

In summary, strong biofilm formation among MRSA isolates is associated with multiple features of the host and organism including phenotypic and genotypic factors, demographics, and clinical characteristics. Patients with a strong biofilm-forming MRSA isolate are 5 times more likely to be admitted or readmitted within 90 days, and tend to have decreased mortality at 90 days.

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Table 1. Baseline Characteristics

Characteristic	Strong Biofilm OD ≥ 2.0 (n=47)	Weak Biofilm OD ≤ 1.0 (n = 74)	*P* value
Age, years [a]	67.8 ± 13.5	68.1 ± 12.8	0.90
Male sex	44 (93.6)	72 (97.3)	0.37
Race - white	44 (93.6)	59 (79.7)	0.04
Residence – home	39 (83.0)	51 (68.9)	0.08
Weight, kg [a]	89.9 ± 23.0	84.4 ± 21.0	0.18
BMI [a]	29.4 ± 7.9	27.1 ± 6.4	0.08
SCr, (mg/dL) [b]	0.9 (0.8-1.1)	1.3 (0.9-2.2)	0.001
CrCl, mL/min [b]	92.6 (67.6-117.6)	58.4 (31.7-89.2)	0.001
Charlson Comorbidity Index [b]	5.0 (3- 8)	5.0 (3-8)	0.91
Comorbidities			
IV Drug User	2 (4.3)	2 (2.7)	0.64
Alcohol Abuse	6 (12.8)	6 (8.1)	0.53
Diabetes	17 (36.2)	35 (47.3)	0.23
Cardiovascular	36 (76.6)	59 (79.7)	0.68
Chronic respiratory disease	14 (29.8)	19 (25.7)	0.62
Liver disease	5 (10.6)	7 (9.5)	1.00
Chronic renal disease	6 (12.8)	23 (31.1)	0.02
Malignancy	14 (29.8)	21 (28.4)	0.87
Anemia	9 (19.2)	24 (32.4)	0.11
Other	13 (27.7)	11 (14.9)	0.08
Smoking Status			0.80
Non-Smoker	23 (48.9)	32 (43.2)	
Smoker	14 (29.8)	23 (31.1)	
Unknown	10 (21.3)	19 (25.7)	
Foreign material/device	12 (25.5)	37 (50.0)	0.01
Orthopedic	2 (4.3)	2 (2.7)	0.01
Other	10 (21.3)	35 (47.3)	
None	35 (74.5)	37 (50.0)	
No. Foreign material/device [b]	0 (0-1)	0.5 (0-1)	0.01
	Patient History		
Hospitalization (≥ 2 days) [c]	13 (27.7)	39 (52.7)	0.007
Surgery [c]	13 (27.7)	18 (24.3)	0.68
Medications [c]			
Chemotherapy/Immunosuppressants	5 (10.6)	2 (2.7)	0.11
Chronic corticosteroids [d]	6 (12.8)	5 (6.8)	0.33
NSAID	19 (40.4)	35 (47.3)	0.46
Gastric acid suppressor [e]	21 (44.7)	44 (59.5)	0.11
HMG-CoA reductase inhibitor	21 (44.7)	28 (37.8)	0.45
Antimicrobial Use [c]	29 (61.7)	57 (77.0)	0.07
Vancomycin	1 (2.1)	20 (27.0)	0.001

Penicillin	9 (19.2)	21 (28.4)	0.25
Cephalosporin	9 (19.2)	19 (25.7)	0.41
Beta-Lactams	14 (29.8)	30 (40.5)	0.23
Fluoroquinolone	11 (23.4)	24 (32.4)	0.29
Other	14 (29.8)	26 (35.1)	0.54
No. Antibiotic [c] [b]	1 (0-2)	1 (0-2)	0.08
Infections [f]			
Skin and soft tissue	5 (10.6)	9 (12.2)	0.80
Pneumonia	5 (10.6)	19 (25.7)	0.04
Urinary tract infection	14 (29.8)	24 (32.4)	0.76
Bacteremia	2 (4.3)	13 (17.6)	0.03
Other	7 (14.9)	13 (17.6)	0.70
≥1 *S. aureus* infection previous [f]	12 (25.5)	21 (28.4)	0.73
MRSA	11 (23.4)	18 (24.3)	0.91
Source of previous *S. aureus* infection [g]			
Tissue	5 (10.6)	3 (4.1)	0.26
Urine	6 (12.8)	5 (6.8)	0.33
Blood	0	5 (6.8)	0.15
Other	3 (6.4)	11 (14.9)	0.15
Index isolate same site as previous *S. aureus* infection	9 (19.2)	12 (16.2)	0.68
Previous Polymicrobial infections	13 (27.7)	23 (31.1)	0.69
MRSA nares positive [f]	6 (12.8)	15 (20.3)	0.29
Index Culture			
Culture Site (%)			
Blood	9 (19.1)	23 (31.1)	0.15
Tissue	16 (34.0)	20 (27.0)	0.41
Urine	11 (23.4)	13 (17.6)	0.43
Catheter	10 (21.3)	15 (20.3)	0.89
Other	1 (2.1)	3 (4.1)	1.0
Bacteremia Source			
Foreign material	3 (6.4)	10 (13.5)	0.22
cSSTI/Osteomyelitis	0	4 (5.4)	0.16
Other	6 (12.8)	16 (21.6)	0.22
Trauma associated	5 (10.6)	9 (12.2)	0.80
At Index Culture			
Setting			0.04
Inpatient	23 (48.9)	50 (67.6)	
Outpatient	24 (51.1)	24 (32.4)	
Inpatient admission			0.70
ICU	6 (26.1)	11 (22.0)	
Non-ICU	17 (73.9)	39 (78.0)	
Length of stay, days [b]	14.0 (4.0-28.0)	12.5 (7.0-20.0)	0.47

Surgery/procedure during admission	13 (27.7)	32 (43.2)	0.08
Hospital days prior to index culture [b]	0 (0-3)	0 (0-2)	0.85
MRSA nares positive	10 (21.3)	26 (35.1)	0.10
Urinary foley catheter	18 (38.3)	27 (36.5)	0.84
IV catheter > 48 hours	6 (12.8)	20 (27.0)	0.06
Mechanical ventilation	3 (6.4)	7 (9.5)	0.74
Dialysis	0	10 (13.5)	0.006

Data are presented as No. (%) unless otherwise specified.
Abbreviations: BMI, body mass index; SCr, Serum creatinine; CrCl, creatinine clearance (Cockcroft-gault); cSSTI, complicated skin and soft tissue infection; ICU, intensive care unit; IV, intravenous;
[a] Mean ± SD
[b] Median (Q1-Q3)
[c] Previous 90 days
[d] Prednisone 20 mg every day or equivalent for ≥ 14 days
[e] Proton-pump inhibitor or H_2-antagonists
[f] Previous one year
[g] ≥ 1 previous infection source

Table 2. Phenotypic and Genotypic Characteristics

Phenotypic	**Strong Biofilm OD ≥ 2.0 (n=47)**	**Weak Biofilm OD ≤ 1.0 (n=74)**	***P* value**
Alpha-toxin	35 (74.5)	61 (82.4)	0.29
Beta-toxin	28 (59.6)	56 (75.7)	0.06
Agr operon dysfunction (delta-toxin negative)	29 (61.7)	32 (43.2)	0.05
hVISA	4 (8.5)	3 (4.4)	0.44
Pigmented	36 (76.6)	40 (54.1)	0.01
Vancomycin MIC			0.37
≥ 1.5 μg/mL	28 (59.6)	50 (67.6)	
< 1.5 μg/mL	19 (40.4)	24 (32.4)	
Genotypic	**Strong Biofilm OD ≥ 2.0 (n=47)**	**Weak Biofilm OD ≤ 1.0 (n=74)**	
MLST CC			
CC 5	23 (48.9)	54 (73.0)	0.007
CC 8	16 (34.0)	11 (14.9)	0.01
Other [a]	8 (17.0)	9 (12.2)	0.45
Spa Type			
t002	14 (29.8)	25 (33.8)	0.65
t895	1 (2.1)	18 (24.3)	0.001
t008	12 (25.5)	6 (8.1)	0.01
t1094	4 (8.5)	3 (4.1)	0.43
Other [b]	16 (34.0)	22 (29.7)	0.62

Data are presented as No. (%) unless otherwise specified.
Abbreviations: *Agr,* accessory gene regulator; hVISA, heteroresistant vancomycin intermediate *S. aureus*; MLST CC, multi-locus sequence typing clonal complex
[a] CC1, CC4, CC20, CC30, CC45, and unable to obtain genotypic characteristics (eleven isolates)
[b] t004, t010, t018, t062, t064, t067, t088, t1340, t189, t1904, t2032, t242, t2666, t334, t548, t681, t693, t985, and unable to obtain genotypic characteristics (eleven isolates)

Table 3. Clinical Outcomes

Outcome	No. of events/No. of patients (%)			Unadjusted	Adjusted
	Strong Biofilm	Weak Biofilm	*P* Value	OR (95% CI)	OR (95% CI)
Mortality					
30-day	3/47 (6.4)	18/74 (24.3)	0.01	0.21 (0.06-0.77)	0.32 (0.08-1.26) [a]
90-day	6/47 (12.8)	27/74 (36.5)	0.004	0.25 (0.10-0.68)	0.36 (0.12-1.06) [a]
(Re)-admission					
30-day	11/45 (24.4)	17/61 (27.9)	0.69	0.84 (0.35-2.02)	1.65 (0.58-4.65) [b]
90-day	20/43 (46.5)	23/57 (40.3)	0.54	1.28 (0.58-2.86)	5.43 (1.69-17.4) [c]
MRSA (Re)-infection					
30-day	3/47 (6.4)	8/66 (12.1)	0.36	0.49 (0.12-1.97)	0.33 (0.08-1.37) [d]
90-day	8/45 (17.8)	14/58 (24.1)	0.43	0.68 (0.26-1.80)	0.74 (0.25-2.18) [e]
MRSA related (Re)-admission					
30-day	5/44 (11.4)	8/61 (13.1)	0.79	0.85 (0.26-2.80)	1.20 (0.34-4.25) [f]
90-day	8/43 (18.6)	9/57 (15.8)	0.71	1.22 (0.43-3.47)	1.75 (0.56-5.45) [f]

Abbreviations: OR, odds ratio; CI, confidence interval; ICU, intensive care unit; BMI, body mass index

[a] Adjusted for hospitalized during previous 90 days for > 2days and admission type (inpatient or outpatient setting)

[b] Adjusted for hospitalized during previous 90 days for > 2days and infection with confirmed bacteremia at the time of index culture

[c] Adjusted for hospitalized during previous 90 days for > 2days, MLSTcc5, serum creatinine and infection with confirmed pneumonia at the time of index culture

[d] Adjusted for pigmentation

[e] Adjusted for MLSTcc5 and pigmentation

[f] Adjusted for hospitalized during previous 90 days for > 2days

Table 4: Predictors of Strong Biofilm Producing MRSA

Variable	OR (95% CI)
Beta-toxin	0.31 (0.11–0.89)
Chemotherapy or immunosuppressants used in previous 90 days	33.6 (1.68–673)
Serum creatinine (per unit increase)	0.33 (0.15–0.72)
Spa type t008	4.54 (1.21-17.1)
Spa type t895	0.02 (<0.001–0.47)
Vancomycin in the previous 90 days	0.03 (0.002–0.39)

Abbreviations: OR, odds ratio; CI, confidence interval