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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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1   **ABSTRACT**

2   **Background.** Molecular and clinical factors associated with biofilm-forming methicillin-resistant

3   *Staphylococcus aureus* (MRSA) are incompletely understood.

4   **Methods.** Biofilm production was quantified in 182 MRSA isolates from clinical culture sites

5   (2004-2013). Microbiologic toxins, pigmentation, and genotypes were evaluated, and patient

6   demographics were collected. Logistic regression was used to quantify the effect of strong biofilm

7   production (versus weak) on clinical outcomes and independent predictors of strong biofilm.

8   **Results.** Of isolates evaluated, 25.8% (47/182) produced strong biofilm, and 40.7% (74/182)

9   produced weak biofilm. Strong biofilm-producing isolates were more likely to be from MLST clonal

10   complex 8 (34.0% vs. 14.9%; P=0.01), but less likely to be from MLST CC5 (48.9% vs. 73.0%,

11   P=0.007). Predictors for strong biofilm were *spa* type t008 (aOR 4.54 95%CI 1.21-17.1), and

12   receiving chemotherapy or immunosuppressants in the previous 90 days (aOR 33.6; 95%CI 1.68-

13   673). Conversely, patients with high serum creatinine (aOR 0.33; 95%CI 0.15-0.72) or who

14   previously received vancomycin (aOR 0.03; 95%CI 0.002-0.39) were less likely to harbor strong

15   biofilm-producing MRSA. Beta-toxin producing isolates (aOR 0.31; 95%CI 0.11-0.89) and isolates

16   with *spa* type t895 (aOR 0.02 95%CI <0.001-0.47) were less likely to produce strong biofilm.

17   Patient outcomes also varied between the two groups. Specifically, patients with strong biofilm-

18   forming MRSA were significantly more likely to be readmitted within 90 days (aOR 5.43; 95%CI

19   1.69-17.4), but tended to have decreased 90-day mortality (aOR 0.36; 95%CI 0.12-1.06).

20   **Conclusions:** Patients that harbored t008 and received immunosuppressants were more likely

21   to have a strong biofilm-producing MRSA. Clinically, patients with strong biofilm-forming MRSA

22   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

49   to October 2013.   Nares swabs collected for infection control surveillance purposes were

50   excluded. Duplicate isolates with the same multi-locus sequence typing (MLST) clonal complexes

51   (CC) and collected from the same date or admission were excluded. Each isolate included was

52   treated as an independent event, and therefore patients may have been included in the study

53   more than once. This study was approved by the Institutional Review Board and the Research

54   and Development Committee of the PVAMC.

55

56   **Microbiological (phenotypic and genotypic) data.**

57   ***Biofilm formation assay***.   Biofilm formation was determined using a modified Christensen

58   method as previously described by our group.(12-16) *Staphylococcus epidermidis* ATCC 35984

59   and methicillin-susceptible *Staphylococcus aureus* ATCC 35556 were used as positive controls.

60   The isogenic accumulation-negative mutant of ATCC 35984, M7, was used as a negative

61   control.(17-19) After incubation, planktonic bacteria were removed by rinsing each well three

62   times with sterile Millipore water. Plates were dried overnight then stained with 0.1% crystal violet

63   for 15 minutes. Adherent stain was resolubilized with 33% glacial acetic acid for one hour before

64   measuring optical density (OD) at 570 nm on a spectrophotometer (ELX800, Biotek, Winooski,

65   VT). To obtain the final OD values the OD of wells containing tryptic soy broth (TSB) with 1.0%

66   dextrose only (media control) were subtracted from wells containing isolates to remove

67   background readings.   Mean OD was calculated for each isolate, using at least four

68   replicates.(17, 20) We used the degree of biofilm production; strong (OD ≥ 2.0), moderate (1.0 <

69   OD < 2.0) and weak (OD ≤ 1.0) as previously described.(21)   For this study, we excluded

70   moderate biofilm-producing isolates.

71

72   ***Alpha- and Beta-toxin production.*** Qualitative alpha-toxin production, indicated by clear zones

73   of hemolysis, was evaluated for each strain on Mueller-Hinton agar with 5% sheep blood after

74 24h incubation at 37°C.(22) Plates were then refrigerated at 4°C for 24h to evaluate beta-toxin

75 production, indicated by green-brown hemolysis.

76

77 ***Determination of Agr operon function.*** Function of the *agr* operon was measured qualitatively

78 by delta-toxin production.(22, 23) Delta-toxin expression was determined by streaking the MRSA

79 test isolates adjacent to a Beta Lysin Disk (Remel, Lenexa, KS) on tryptic soy agar with 5% sheep

80 blood and incubated at 37°C for 24 hours. The presence of synergistic hemolysis between the

81 streak and Beta Lysin Disk indicated the production of delta-hemolysis, therefore a functional *agr*

82 locus.(22, 23) The dysfunction of *agr* was defined as the absence of delta-hemolysis within the

83 beta-toxin zone, as evidenced by the lack of synergistic hemolysis.(23) Reference strains

84 RN4420 and RN6607 were used as negative and positive controls for delta-toxin, respectively.

85

86 ***Heterogeneous vancomycin intermediate Staphylococcus aureus (hVISA) presence.***

87 Screening for hVISA was conducted using E-test glycopeptide resistance detection (GRD) strips

88 (bioMérieux, Durham, NC).(24) Testing was conducted according to manufacturer's instructions

89 using a standard 0.5 McFarland bacterial suspension on Mueller-Hinton agar with 5% sheep blood

90 (BD, Sparks, MD). The results were read at 24 and 48 hours after incubation. Standard

91 vancomycin Etests were also conducted according to manufacturer's instructions, on Mueller-

92 Hinton agar for 24 hours. Heteroresistance was defined as a vancomycin or teicoplanin MIC of ≥

93 8 µg/mL on the GRD Etest plus a standard vancomycin MIC < 4 µg/mL. Quality control of

94 susceptibility testing was performed with reference strain ATCC700698 (Mu3, hVISA).

95

96 ***Pigmentation.*** Golden pigmentation was evaluated after overnight growth on tryptic soy agar at

97 37°C.(25, 26) Each strain was compared to a reference white strain of *S. epidermidis* ATCC35984

98 and categorized as pigmented or non-pigmented. *S. aureus* ATCC35556 served as a pigmented

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

100   98.3% agreement.

101

102   *Genotyping*. Staphylococcal protein A (*spa*) genotype was determined by PCR as previously

103   described, with 1095F and 1517R primers.(27) Gene sequences were determined using Sanger

104   sequencing with the forward primer only unless reverse primer was necessary for sequence

105   clarification. *Spa* type was mapped to common MLST CC using the Ridom spa server

106   (Spaserver.ridom.de). *Spa* types not matched to a clonal complex in the Ridom spa server were

107   matched by literature search.

108

109   **Patient data**. Data was collected through a chart review of electronic medical records and

110   included diagnoses and procedures, clinical measurements, microbiology data, patient

111   demographics, health-care exposure within 90 days of index culture (hospitalization >72 hours

112   and surgical procedures), receipt of antimicrobials or medications that may influence biofilm

113   formation in the previous 90 days (i.e. gastric acid suppressants [proton-pump inhibitors or H_2-

114   blockers], chronic corticosteroid use, non-steroidal anti-inflammatory [NSAID], HMG-CoA

115   reductase inhibitors [statins])(28-32), presence of prosthetic/foreign devices (i.e. orthopedic,

116   cardiovascular, urinary Foley, intravenous catheters), and infection/colonization history in the

117   previous year.

118

119   **Clinical outcome definitions**. Clinical outcomes of interest were all-cause mortality, admission

120   among outpatient or re-admission among inpatients, MRSA infection and MRSA-related

121   admission among outpatients or re-admission among inpatients. As the risk period for poor

122   outcomes in these patients is not known, we evaluated outcomes at follow-up periods of 30 and

123   90 days.

124

125   The index date was defined as the collection date of the MRSA isolate tested for biofilm production

126   (index culture). MRSA infection was confirmed from microbiology data, and diagnosis of infection

127   in the medical record. Readmission was defined as admission for any reason after the discharge

128   date of the index culture. For index isolates collected in the outpatient setting, admission was

129   defined as admission for any reason after the index date.

130

131   **Statistical analysis**. Between-group differences were assessed using X^2 or Fisher exact tests

132   for categorical variables and the T-test or Wilcoxon rank sum test for continuous variables.

133   Logistic regression models were used to quantify the effect of strong biofilm on each clinical

134   outcome, while controlling for confounders of the exposure-outcome relationship.(33)   In

135   multivariable modeling, a manual, non-computer-generated backward elimination approach was

136   implemented. Logistic regression was also used to identify independent predictors associated

137   with strong MRSA biofilm production.(33)   All baseline variables were evaluated as potential

138   confounders in the clinical outcome models, and as independent predictors of biofilms in the

139   predictive model. Crude and adjusted odds ratios (OR) and respective 95% confidence intervals

140   (CI) are presented. All statistical tests were conducted using SAS, version 9.2 (SAS Institute,

141   Cary, NC), with a two-tailed α value of 0.05 required for statistical significance.

142

143 **RESULTS**

144 **Isolate and clinical characteristics.** In total, 121 MRSA isolates were included for biofilm

145 production, 38.8% (47/121) produced strong biofilms (OD ≥ 2.0) and 61.2% (74/121) produced

146 weak biofilms (OD ≤ 1.0). Race was significantly different between the groups, with the strong

147 biofilm group having a higher number of whites (93.6% vs. 79.7%; P=0.04). There was no

148 difference between the groups in age, gender or BMI. Serum creatinine and creatinine clearance

149 was significantly different between the two groups. Median (Q1-Q3) serum creatinine was 0.9 (0.8

150 - 1.1mg/dl) for the strong biofilm group vs 1.3 (0.9 - 2.2 mg/dL) for the weak biofilm group

151 (P=0.001). The median (Q1 – Q3) creatinine clearance was 92.6 (range 67.6-117.6 mL/min) in

152 the strong biofilm group vs 58.4 (31.7 – 89.2 mL/min) for the weak biofilm group (P=0.001).

153 Significantly lower number of patients in the strong biofilm group had chronic renal failure (12.8%

154 vs 31.1%; P=0.02). There was no difference between the groups in Charlson comorbidity index

155 or other comorbidities such as diabetes, cardiovascular disease, liver disease, malignancies and

156 anemia. The groups did not differ in IV drug use, alcohol abuse or smoking. The presence of

157 foreign material/device was lower in patients with a strong biofilm-producing isolate (25.5% vs

158 50.0%; P=0.01). Significantly lower number of patients in the strong biofilm group were

159 hospitalized for two or more days in the previous 90 days (27.7% vs 52.7%; P=0.007). Overall

160 antimicrobial use in the previous 90 days from culture was not significantly different between the

161 groups but use of vancomycin was significant with 27.0% of patients receiving vancomycin in the

162 weak biofilm group whereas only 2.1% in the strong biofilm group (P=0.001). There were fewer

163 patients on hemodialysis in the strong biofilm-producing group (0% vs. 13.5%; P=0.006). Patients

164 in the strong biofilm group had a lower number of bacteremias (4.3% vs 17.6%; P=0.03) and

165 pneumonias (10.6% vs 25.7%; P=0.04) in the year prior to the index culture. Patients in the strong

166 biofilm group tended to present in the outpatient setting at the index culture (51.1% vs 32.4%;

167 P=0.04). **(Table 1)**.

168

169   Alpha-toxin was produced by 79.3% (n=96) of the isolates overall (74.5% strong biofilm vs

170   82.4%weak biofilm, p=0.29). Beta-toxin production was less common, with 69.4% (n=84) of

171   isolates (59.6% strong biofilm vs 75.7% weak biofilm, p=0.06). Presence of hVISA was rare

172   among strong biofilm and weak biofilm-producing isolates (8.5% vs 4.4%; P=0.44).   The

173   proportion of isolates with *agr* dysfunction (61.7% vs 43.2%; P=0.05) and pigmentation (76.6%

174   vs 54.1%; P=0.01) were significantly higher in the strong biofilm group. The distribution of

175   vancomycin MIC was similar among both groups. MRSA isolates represented seven MLST CC,

176   the most common were CC5 (63.6%) and CC8 (22.3%). Strong biofilm-producing isolates had

177   significantly lower MLST CC5 (48.9% vs 73.0%; P=0.007) and significantly higher CC8 (34.0% vs

178   14.9%; P=0.01). There were 24 different *spa* types identified among the isolates. Of those *spa*

179   types, the most common were t002 (32.2%), t895 (15.7%), t008 (14.9%), and t1094 (5.8%).

180   Strong biofilm-producing isolates contained significantly more *spa* type t008 (25.5% vs 8.1%;

181   P=0.01) and less t895 (2.1% vs 24.3%; P=0.001). **(Table 2)**.

182

183   **Clinical Outcomes and Independent Predictors.** After controlling for potential confounders,

184   patients with strong biofilm-producing MRSA were more than five times as likely to be (re)-

185   admitted within 90 days of discharge (adjusted OR 5.43; 95% CI 1.69-17.4). The strong biofilm

186   group was 64% less likely to die within 90 days (adjusted OR 0.36; 95% CI 0.12-1.06), but this

187   was not statistically significant. There was no difference in 30 day mortality, 30 day (re)-admission,

188   MRSA reinfection at 30 or 90 days, or MRSA related (re)-admission at 30 or 90 days among

189   patients with strong or weak biofilm-producing MRSA. (**Table 3)**

190

191   Patients who were on chemotherapy and/ or used immunosuppressants within 90 days of index

192   culture had a 33.6 times higher odds for strong biofilm-producing MRSA isolate (adjusted OR

193   33.6; 95% CI 1.68-673). Patients harboring isolates from t008 (adjusted OR 4.54; 95% CI 1.21-

194   17.1) also had increased risk of a strong biofilm-producing MRSA. Further, patients with isolates

195   from t895 (adjusted OR 0.02; 95% CI <0.001-0.47) or that produced beta-toxin were less likely to

196   produce strong biofilm (adjusted OR 0.31; 95%CI 0.11-0.89). Patients who had increased serum

197   creatinine (adjusted OR 0.33; 95% CI 0.15-0.72) or who received vancomycin in the previous 90

198   days (adjusted OR 0.03; 95%CI 0.002-0.39) were less likely to produce strong biofilm. **(Table 4)**.

199

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

226 in all categories. This may represent a higher severity of illness, and may help to explain the

227 increased mortality seen at 90 days. In contrast, CC8, the traditionally community-acquired clone,

228 has been previously associated with strong biofilm production, as well as community-acquired

229 skin infections and colonization.(34, 37) These types of infections and colonization may be

230 associated with lower mortality, as seen in our study. The most common *spa* types were t002,

231 t008, t895, and t1094. Though t002 and t895 are related to CC5, t002 was not related to biofilm

232 formation. We found that isolates from *spa* type t008 were predictive for the strong biofilm

233 phenotype, while t895 was significantly higher in weak biofilm-producing isolates. At least for this

234 subset of isolates, *spa* type served as a better predictor of biofilm formation than MLST CC,

235 potentially due to the greater degree of resolution in *spa* typing. This finding is consistent with

236 previous studies evaluating genotypically different clones of MRSA in the production of biofilm.(6,

237 7)

238

239 Previously published data suggest that *agr* dysfunction is associated with biofilm formation in *S.*

240 *aureus*.(5, 38-40) This is in line with our own data, which demonstrated *agr* dysfunction was

241 present in 61.7% of strong biofilm formers versus 43.2% of weak biofilm formers. Some data

242 demonstrate conflicting results with regard to *agr* function and biofilm, depending whether the

243 biofilm is formed in vivo or in vitro.(41) In vitro biofilm formation may yield a different relationship

244 with *agr* than in vivo biofilms, since there is no host response-relationship. It is suggested that

245 the host response and *agr*-dependent virulence factors secreted in vivo regulate biofilm

246 formation.(41) Previous studies have also suggested that *agr* dysfunction is associated with

247 hVISA development, however because our overall numbers of hVISA were low, we could not

248 confirm this finding.(22, 42) Beta-toxin was associated with weak biofilm formation, and was a

249 negative predictor for strong biofilm in the logistic regression model (adjusted OR 0.31; 95%CI

250 0.11-0.89). Although there is limited data on the connection between beta-toxin and biofilm

251 formation, in previous studies, beta-toxin was associated with skin colonization, and colonization

252 was associated with a low biofilm phenotype, consistent with our findings.(43, 44) Alpha-toxin has

253 also been associated with biofilm formation,(45, 46) however, we did not quantify how much

254 alpha-toxin these isolates produce in this study, which may have correlated better to biofilm

255 formation than a dichotomous presence or absence of alpha-toxin. Overall, these findings

256 underscore the need for additional studies to better describe the mechanisms responsible for the

257 presence of biofilms.

258

259 This study had several limitations. A limited sample size may have impaired the ability to find

260 associations between biofilm production and covariates previously noted to play a role in biofilms.

261 Of course, we cannot guarantee that in vitro biofilm formation equates to clinical biofilm formation

262 in an infection. Due to the retrospective design of this study, not all variables or potential

263 confounders may have been included in the analysis of clinical factors, and we are reliant on the

264 accuracy of data as entered into the patient electronic medical record. To minimize selection bias,

265 the investigator collecting clinical data was blinded to the biofilm status of each isolate. Biofilm

266 formation is determined using a standard assay.(14, 15, 18, 19, 47) Additionally, we utilize a

267 negative control isolate to ensure comparability between results. By removing the moderate

268 biofilm category, we may have limited our power in the number of isolates, but the isolates had

269 the most different biofilm classifications to see differences in the predictors and outcomes.

270

271 In summary, strong biofilm formation among MRSA isolates is associated with multiple features

272 of the host and organism including phenotypic and genotypic factors, demographics, and clinical

273 characteristics. Patients with a strong biofilm-forming MRSA isolate are 5 times more likely to be

274 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 OR (95% CI)	Adjusted OR (95% CI)
	Strong Biofilm	**Weak Biofilm**	*P* Value		
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