

2018

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

Megan Luther
University of Rhode Island

Diane M. Parente
University of Rhode Island

See next page for additional authors

Follow this and additional works at: https://digitalcommons.uri.edu/php_facpubs

**The University of Rhode Island Faculty have made this article openly available.
Please let us know how Open Access to this research benefits you.**

This is a pre-publication author manuscript of the final, published article.

Terms of Use

This article is made available under the terms and conditions applicable towards Open Access Policy Articles, as set forth in our [Terms of Use](#).

Citation/Publisher Attribution

Luther MK, Parente DM, Caffrey AR, Daffinee KE, Lopes VV, Martin ET, LaPlante KL. 2018. Clinical and genetic risk factors for biofilm-forming *Staphylococcus aureus*. *Antimicrob Agents Chemother*62:e02252-17. doi: 10.1128/AAC.02252-17
Available at: <http://dx.doi.org/10.1128/AAC.02252-17>

This Article is brought to you for free and open access by the Pharmacy Practice at DigitalCommons@URI. It has been accepted for inclusion in Pharmacy Practice Faculty Publications by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons@etal.uri.edu.

Authors

Megan Luther, Diane M. Parente, Aisling R. Caffrey, Katie Daffinee, Vrishali V. Lopes, Emily T. Martin, and Kerry L. LaPlante

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

Megan K. Luther,^{1,2,3} Diane M. Parente,^{1,2} Aisling R. Caffrey,^{1,2,3,4} Katie Daffinee,¹ Vrishali V. Lopes,¹ Emily T. Martin,⁵ Kerry L. LaPlante,^{1,2,3,4}

1. Rhode Island Infectious Diseases Research Program, Veterans Affairs Medical Center, Providence, Rhode Island,
2. College of Pharmacy, University of Rhode Island, Kingston, Rhode Island,
3. Veterans Affairs Medical Center, Center of Innovation in Long Term Services and Supports, Providence, Rhode Island,
4. Brown University, Providence, Rhode Island,
5. University of Michigan School of Public Health, Ann Arbor, Michigan,

Running Head: Genetic and Clinical Risks of MRSA Biofilms

Address Correspondence: Kerry L. LaPlante, Pharm.D., Professor, University of Rhode Island, College of Pharmacy, 7 Greenhouse Rd, Suite 295A, Kingston, RI 02881, 401-874-5560 (office); KerryLaPlante@uri.edu

Word Count: 2939

Keywords: Biofilm, *S. aureus*, multi-locus sequence type medical outcomes,

Version Date: February 23, 2018

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.

23 **BACKGROUND**

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

32
33 Methicillin-resistant *S. aureus* (MRSA) causes significant morbidity and mortality. Risk factors for
34 infection with MRSA are clearly defined; however, little is known about the molecular and clinical
35 risk factors for biofilm-producing MRSA.(8-11) Defining these risk factors and understanding the
36 clinical outcomes associated with biofilm-producing MRSA can provide critical and timely insight
37 into the prevention and treatment of these serious infections. Further, understanding the
38 phenotypic and genetic characteristics associated with biofilms in MRSA may enable the
39 development of biofilm detection methods in clinical microbiology laboratories and identify
40 therapeutic targets. Therefore, the objectives of this study were to quantify clinical outcomes
41 among adult patients with strong biofilm-producing MRSA ($OD \geq 2.0$) or weak biofilm-producing
42 MRSA ($OD \leq 1.0$) and to identify clinical and molecular independent predictors of strong biofilm-
43 producing MRSA.

44

45 **MATERIALS AND METHODS**

46 **Study design, population, and bacterial isolates.** A retrospective cohort study was conducted
47 among a sample of inpatient and outpatients with MRSA cultures from any culture site at the
48 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 ($OD \geq 2.0$), moderate ($1.0 <$
69 $OD < 2.0$) and weak ($OD \leq 1.0$) 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 \geq
93 8 $\mu\text{g/mL}$ on the GRD Etest plus a standard vancomycin MIC $< 4 \mu\text{g/mL}$. 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 H₂-
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 χ^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 ($OD \geq 2.0$) and 61.2% (74/121) produced
146 weak biofilms ($OD \leq 1.0$). 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

200 **DISCUSSION**

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

205
206 Biofilm formation has been previously associated with patient mortality. A previous study
207 demonstrated increased mortality with biofilm-forming isolates, but the attributable mortality was
208 low.(3) Similar to our study, included patients were primarily male, and members of military
209 services (however they were younger than our Veterans), but whereas our study was only in
210 MRSA isolates, this study included multiple types of bacterial cultures and found a five-fold
211 increased association of MRSA among the biofilm-positive group (OR 5.09, 95% CI 1.12-23.1).
212 Overall mortality with initial infection was 16% versus 5% in biofilm versus non-biofilm group
213 ($p=0.01$), with an attributable mortality of 7%.(3) Unfortunately, it is difficult to tell how many of
214 these are due to biofilm-forming versus non-biofilm forming MRSA in the study, as opposed to
215 other bacterial types.

216
217 The majority of MRSA isolates in our study represented CC5, typically referred to as hospital-
218 associated strains and CC8 historically of community origin. In the multivariate analyses, there
219 was no association between clonal complex and biofilm formation, which has been found in other
220 studies.(34-36) This may be due to limited number of isolates, or the clinical source of the isolates
221 used, which may play a role in their biofilm formation. However, in univariate analyses, weak
222 biofilm isolates had more CC5, which is traditionally hospital-associated, as well as more previous
223 hospitalization within the previous 90 days, dialysis, bacteremia and pneumonia within the
224 previous year, and treatment with vancomycin. Although not statistically significant, weak biofilm
225 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.

275 **FUNDING INFORMATION**

276 The views expressed in this article are those of the authors and do not necessarily reflect the
277 position or policy of the United States Department of Veterans Affairs. This research was
278 supported, in part upon work conducted using the Rhode Island Genomics and Sequencing
279 Center which is supported in part by the National Science Foundation [EPSCoR Grants Nos.
280 0554548 and EPS-1004057], the Office of Academic Affiliations of the Department of Veterans
281 Affairs [to Diane M. Parente and Megan Luther], and National Institutes of Health/National
282 Institute of Allergy and Infectious Diseases [K01 A109906 to Emily T. Martin]. The funders had
283 no role in study design, data collection and interpretation, or the decision to submit the work for
284 publication.

285

286 **ACKNOWLEDGEMENTS**

287 We gratefully acknowledge Simon Sarkisian, Jeffrey Coleman, Ann Sam, Janet Atoyan, and
288 Elizabeth Salzman for assay assistance and interpretation. M.K.L. has received research funding
289 from Pfizer Inc. and Merck Pharmaceuticals. D.M.P., K.E.D., V.V.L. no conflicts. A.R.C. has
290 received research funding from Pfizer, Merck, and The Medicines Company. E.T.M. has received
291 research funding from Pfizer Inc., Merck, and Sage Therapeutics. K.L.L. has received research
292 funding or served as an advisor or consultant for Allergan, BARD/Davol, Merck, The Medicines
293 Company, Ocean Spray, Achaogen, Zavante, and Pfizer.

REFERENCES

1. Cha JO, Yoo JI, Yoo JS, Chung HS, Park SH, Kim HS, Lee YS, Chung GT. 2013. Investigation of Biofilm Formation and its Association with the Molecular and Clinical Characteristics of Methicillin-resistant *Staphylococcus aureus*. *Osong Public Health Res Perspect* 4:225-32.
2. Wang L, Yu F, Yang L, Li Q, Zhang X, Zeng Y, al. e. 2010. Prevalence of virulence genes and biofilm regulation in *Staphylococcus aureus* isolates from device-related infections. *Afr J Microbiol Res*:2566-2569.
3. Barsoumian AE, Mende K, Sanchez CJ, Jr., Beckius ML, Wenke JC, Murray CK, Akers KS. 2015. Clinical infectious outcomes associated with biofilm-related bacterial infections: a retrospective chart review. *BMC Infect Dis* 15:223.
4. Swarnakar M, Tiwari K, Banerjee T. 2013. Study of biofilm formation in gram positive clinical isolates and associated risk factors. *Int J Pharm Bio Sci* 4:(B)203-208.
5. Vuong C, Saenz HL, Gotz F, Otto M. 2000. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J Infect Dis* 182:1688-93.
6. Atshan SS, Shamsudin MN, Lung LT, Sekawi Z, Ghaznavi-Rad E, Pei CP. 2012. Comparative characterisation of genotypically different clones of MRSA in the production of biofilms. *J Biomed Biotechnol* 2012:417247.
7. Croes S, Deurenberg RH, Boumans ML, Beisser PS, Neef C, Stobberingh EE. 2009. *Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S. aureus* lineage. *BMC Microbiol* 9:229.
8. Topeli A, Unal S, Akalin HE. 2000. Risk factors influencing clinical outcome in *Staphylococcus aureus* bacteraemia in a Turkish University Hospital. *Int J Antimicrob Agents* 14:57-63.
9. Weber SG, Gold HS, Hooper DC, Karchmer AW, Carmeli Y. 2003. Fluoroquinolones and the risk for methicillin-resistant *Staphylococcus aureus* in hospitalized patients. *Emerg Infect Dis* 9:1415-22.
10. Marshall C, Wolfe R, Kossmann T, Wesselingh S, Harrington G, Spelman D. 2004. Risk factors for acquisition of methicillin-resistant *Staphylococcus aureus* (MRSA) by trauma patients in the intensive care unit. *J Hosp Infect* 57:245-52.
11. Luzar MA, Coles GA, Faller B, Slingeneyer A, Dah GD, Briat C, Wone C, Knefati Y, Kessler M, Peluso F. 1990. *Staphylococcus aureus* nasal carriage and infection in patients on continuous ambulatory peritoneal dialysis. *N Engl J Med* 322:505-9.

12. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH. 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* 22:996-1006.
13. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* 40:175-9.
14. LaPlante KL, Mermel LA. 2007. In vitro activity of daptomycin and vancomycin lock solutions on staphylococcal biofilms in a central venous catheter model. *Nephrol Dial Transplant* 22:2239-46.
15. LaPlante KL, Mermel LA. 2009. In vitro activities of telavancin and vancomycin against biofilm-producing *Staphylococcus aureus*, *S. epidermidis*, and *Enterococcus faecalis* strains. *Antimicrob Agents Chemother* 53:3166-9.
16. LaPlante KL, Woodmansee S. 2009. Activities of daptomycin and vancomycin alone and in combination with rifampin and gentamicin against biofilm-forming methicillin-resistant *Staphylococcus aureus* isolates in an experimental model of endocarditis. *Antimicrob Agents Chemother* 53:3880-6.
17. Schumacher-Perdreau F, Heilmann C, Peters G, Gotz F, Pulverer G. 1994. Comparative analysis of a biofilm-forming *Staphylococcus epidermidis* strain and its adhesion-positive, accumulation-negative mutant M7. *FEMS Microbiol Lett* 117:71-8.
18. Luther MK, Bilida S, Mermel LA, LaPlante KL. 2015. Ethanol and Isopropyl Alcohol Exposure Increases Biofilm Formation in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect Dis Ther* 4:219-26.
19. Luther MK, Mermel LA, LaPlante KL. 2017. Comparison of linezolid and vancomycin lock solutions with and without heparin against biofilm-producing bacteria. *Am J Health Syst Pharm* 74:e193-e201.
20. Polonio RE, Mermel LA, Paquette GE, Sperry JF. 2001. Eradication of biofilm-forming *Staphylococcus epidermidis* (RP62A) by a combination of sodium salicylate and vancomycin. *Antimicrob Agents Chemother* 45:3262-6.
21. Mohamed JA, Huang W, Nallapareddy SR, Teng F, Murray BE. 2004. Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. *Infect Immun* 72:3658-63.
22. Sakoulas G, Eliopoulos GM, Moellering RC, Jr., Wennersten C, Venkataraman L, Novick RP, Gold HS. 2002. Accessory gene regulator (*agr*) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. *Antimicrob Agents Chemother* 46:1492-502.

23. Schweizer ML, Furuno JP, Sakoulas G, Johnson JK, Harris AD, Shardell MD, McGregor JC, Thom KA, Perencevich EN. 2011. Increased mortality with accessory gene regulator (*agr*) dysfunction in *Staphylococcus aureus* among bacteremic patients. *Antimicrob Agents Chemother* 55:1082-7.
24. Leonard SN, Rossi KL, Newton KL, Rybak MJ. 2009. Evaluation of the Etest GRD for the detection of *Staphylococcus aureus* with reduced susceptibility to glycopeptides. *J Antimicrob Chemother* 63:489-92.
25. Lan L, Cheng A, Dunman PM, Missiakas D, He C. 2010. Golden pigment production and virulence gene expression are affected by metabolisms in *Staphylococcus aureus*. *J Bacteriol* 192:3068-77.
26. Lee AC, Bergdoll MS. 1985. Spontaneous occurrence of *Staphylococcus aureus* mutants with different pigmentation and ability to produce toxic shock syndrome toxin 1. *J Clin Microbiol* 22:308-9.
27. Harmsen D, Claus H, Witte W, Rothganger J, Claus H, Turnwald D, Vogel U. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *J Clin Microbiol* 41:5442-8.
28. Alem MA, Douglas LJ. 2004. Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans*. *Antimicrob Agents Chemother* 48:41-7.
29. El-Mowafy SA, Abd El Galil KH, El-Messery SM, Shaaban MI. 2014. Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in *Pseudomonas aeruginosa*. *Microb Pathog* 74:25-32.
30. Goggin R, Jardeleza C, Wormald PJ, Vreugde S. 2014. Corticosteroids directly reduce *Staphylococcus aureus* biofilm growth: an in vitro study. *Laryngoscope* 124:602-7.
31. Graziano TS, Cuzzullin MC, Franco GC, Schwartz-Filho HO, de Andrade ED, Groppo FC, Cogo-Muller K. 2015. Statins and Antimicrobial Effects: Simvastatin as a Potential Drug against *Staphylococcus aureus* Biofilm. *PLoS One* 10:e0128098.
32. Singh V, Arora V, Alam MJ, Garey KW. 2012. Inhibition of biofilm formation by esomeprazole in *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Antimicrob Agents Chemother* 56:4360-4.
33. Hosmer DW, Lemeshow S. 2000. *Applied Logistic Regression*, 2 ed. John Wiley & Sons, Inc., New York, NY.
34. Naicker PR, Karayem K, Hoek KG, Harvey J, Wasserman E. 2016. Biofilm formation in invasive *Staphylococcus aureus* isolates is associated with the clonal lineage. *Microb Pathog* 90:41-9.

35. Jotic A, Bozic DD, Milovanovic J, Pavlovic B, Jesic S, Pelemis M, Novakovic M, Cirkovic I. 2016. Biofilm formation on tympanostomy tubes depends on methicillin-resistant *Staphylococcus aureus* genetic lineage. *Eur Arch Otorhinolaryngol* 273:615-20.
36. Cirkovic I, Knezevic M, Bozic DD, Rasic D, Larsen AR, Dukic S. 2015. Methicillin-resistant *Staphylococcus aureus* biofilm formation on dacryocystorhinostomy silicone tubes depends on the genetic lineage. *Graefes Arch Clin Exp Ophthalmol* 253:77-82.
37. Albrecht VS, Limbago BM, Moran GJ, Krishnadasan A, Gorwitz RJ, McDougal LK, Talan DA, EMERGENCY ID NET Study Group. 2015. *Staphylococcus aureus* Colonization and Strain Type at Various Body Sites among Patients with a Closed Abscess and Uninfected Controls at U.S. Emergency Departments. *J Clin Microbiol* 53:3478-84.
38. Beenken KE, Mrak LN, Griffin LM, Zielinska AK, Shaw LN, Rice KC, Horswill AR, Bayles KW, Smeltzer MS. 2010. Epistatic relationships between *sarA* and *agr* in *Staphylococcus aureus* biofilm formation. *PLoS One* 5:e10790.
39. Yarwood JM, Bartels DJ, Volper EM, Greenberg EP. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. *J Bacteriol* 186:1838-50.
40. Valour F, Rasigade JP, Trouillet-Assant S, Gagnaire J, Bouaziz A, Karsenty J, Lacour C, Bes M, Lustig S, Benet T, Chidiac C, Etienne J, Vandenesch F, Ferry T, Laurent F, Lyon BJI Study Group. 2015. Delta-toxin production deficiency in *Staphylococcus aureus*: a diagnostic marker of bone and joint infection chronicity linked with osteoblast invasion and biofilm formation. *Clin Microbiol Infect* 21:568 e1-11.
41. Kavanaugh JS, Horswill AR. 2016. Impact of Environmental Cues on Staphylococcal Quorum Sensing and Biofilm Development. *J Biol Chem* 291:12556-64.
42. Harigaya Y, Ngo D, Lesse AJ, Huang V, Tsuji BT. 2011. Characterization of heterogeneous vancomycin-intermediate resistance, MIC and accessory gene regulator (*agr*) dysfunction among clinical bloodstream isolates of *staphylococcus aureus*. *BMC Infect Dis* 11:287.
43. Katayama Y, Baba T, Sekine M, Fukuda M, Hiramatsu K. 2013. Beta-hemolysin promotes skin colonization by *Staphylococcus aureus*. *J Bacteriol* 195:1194-203.
44. Pascolini C, Sinagra J, Pecetta S, Bordignon V, De Santis A, Cilli L, Cafiso V, Prignano G, Capitanio B, Passariello C, Stefani S, Cordiali-Fei P, Ensoli F. 2011. Molecular and immunological characterization of *Staphylococcus aureus* in pediatric atopic dermatitis: implications for prophylaxis and clinical management. *Clin Dev Immunol* 2011:718708.

45. Caiazza NC, O'Toole GA. 2003. Alpha-toxin is required for biofilm formation by *Staphylococcus aureus*. *J Bacteriol* 185:3214-7.
46. Anderson MJ, Lin YC, Gillman AN, Parks PJ, Schlievert PM, Peterson ML. 2012. Alpha-toxin promotes *Staphylococcus aureus* mucosal biofilm formation. *Front Cell Infect Microbiol* 2:64.
47. Luther MK, Mermel LA, LaPlante KL. 2014. Comparison of ML8-X10 (a prototype oil-in-water micro-emulsion based on a novel free fatty acid), taurolidine/citrate/heparin and vancomycin/heparin antimicrobial lock solutions in the eradication of biofilm-producing staphylococci from central venous catheters. *J Antimicrob Chemother* 69:3263-7.

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 <i>S. aureus</i> infection previous ^f	12 (25.5)	21 (28.4)	0.73
MRSA	11 (23.4)	18 (24.3)	0.91
Source of previous <i>S. aureus</i> 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 <i>S. aureus</i> 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 H₂-antagonists

^f Previous one year

^g ≥ 1 previous infection source

Table 2. Phenotypic and Genotypic Characteristics

Phenotypic	Strong Biofilm	Weak Biofilm	P value
	OD ≥ 2.0 (n=47)	OD ≤ 1.0 (n=74)	
Alpha-toxin	35 (74.5)	61 (82.4)	0.29
Beta-toxin	28 (59.6)	56 (75.7)	0.06
<i>Agr</i> 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	Weak Biofilm	
	OD ≥ 2.0 (n=47)	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
<i>Spa</i> 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)
<i>Spa</i> type t008	4.54 (1.21-17.1)
<i>Spa</i> 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