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Clinical and Genetic Risk Factors for Biofilm-Forming *Staphylococcus aureus*

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ABSTRACT The molecular and clinical factors associated with biofilm-forming methicillin-resistant *Staphylococcus aureus* (MRSA) are incompletely understood. Biofilm production for 182 MRSA isolates obtained from clinical culture sites (2004 to 2013) was quantified. Microbiological 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 biofilm production) on clinical outcomes and independent predictors of a strong biofilm. Of the isolates evaluated, 25.8% (47/182) produced strong biofilms and 40.7% (74/182) produced weak biofilms. Strong biofilm-producing isolates were more likely to be from multilocus sequence typing (MLST) clonal complex 8 (CC8) (34.0% versus 14.9%; $P = 0.01$) but less likely to be from MLST CC5 (48.9% versus 73.0%; $P = 0.007$). Predictors for strong biofilms were *spa* type t008 (adjusted odds ratio [aOR], 4.54; 95% confidence interval [CI], 1.21 to 17.1) and receipt of chemotherapy or immunosuppressants in the previous 90 days (aOR, 33.6; 95% CI, 1.68 to 673). Conversely, patients with high serum creatinine concentrations (aOR, 0.33; 95% CI, 0.15 to 0.72) or who previously received vancomycin (aOR, 0.03; 95% CI, 0.002 to 0.39) were less likely to harbor strong biofilm-producing MRSA. Beta-toxin-producing isolates (aOR, 0.31; 95% CI, 0.11 to 0.89) and isolates with *spa* type t895 (aOR, 0.02 95% CI, <0.001 to 0.47) were less likely to produce strong biofilms. 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 to 17.4) but tended to have decreased 90-day mortality (aOR, 0.36; 95% CI, 0.12 to 1.06). Patients that harbored t008 and received immunosuppressants were more likely to have strong biofilm-producing MRSA isolates. Clinically, patients with strong biofilm-forming MRSA were less likely to die at 90 days but five times more likely to be readmitted.

KEYWORDS biofilms, *Staphylococcus aureus*, medical outcomes, multilocus sequence type

Biofilms are critical for the pathogenicity of most bacteria, including *Staphylococcus*. As a result, *Staphylococcus 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 the various assays used, 43 to 88% of clinical *S. aureus* isolates can form biofilms (1–4). Biofilm formation in *S. aureus* has been associated with several regulatory and virulence factors, such as accessory gene

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regulator (*agr*) downregulation and heteroresistant vancomycin-intermediate susceptibility (5). Genotypic variation among strains may also affect biofilm production, but these relationships have not 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 insights 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 the clinical outcomes among adult patients with strong biofilm-producing MRSA (optical density [OD] $\geq$ 2.0) or weak biofilm-producing MRSA (OD $\leq$ 1.0) and to identify clinical and molecular independent predictors of strong biofilm-producing MRSA.

RESULTS

Isolate and clinical characteristics. In total, 121 MRSA isolates were included for biofilm production; 38.8% (47/121) produced strong biofilms (OD $\geq$ 2.0), and 61.2% (74/121) produced weak biofilms (OD $\leq$ 1.0). Race was significantly different between the groups, with the strong biofilm group having a higher number of white patients (93.6% versus 79.7%; $P = 0.04$). There was no difference between the groups in age, gender, or body mass index (BMI). The serum creatinine concentration and creatinine clearance were significantly different between the two groups. The median serum creatinine concentration was 0.9 mg/dl (first quartile [Q1] to third quartile [Q3], 0.8 to 1.1 mg/dl) in the strong biofilm group, whereas it was 1.3 mg/dl (Q1 to Q3, 0.9 to 2.2 mg/dl) in the weak biofilm group ($P = 0.001$). The median creatinine clearance was 92.6 ml/min (Q1 to Q3, 67.6 to 117.6 ml/min) in the strong biofilm group, whereas it was 58.4 ml/min (Q1 to Q3, 31.7 to 89.2 ml/min) in the weak biofilm group ($P = 0.001$). A significantly lower proportion of patients in the strong biofilm group had chronic renal failure (12.8% versus 31.1% in the weak biofilm group; $P = 0.02$). There was no difference between the groups in the Charlson comorbidity index or other comorbidities, such as diabetes, cardiovascular disease, liver disease, malignancies, and anemia. The groups did not differ in intravenous drug use, alcohol abuse, or smoking. The rate of the presence of a foreign material/device was lower in patients with a strong biofilm-producing isolate (25.5% versus 50.0%; $P = 0.01$). A significantly lower number of patients in the strong biofilm group had been hospitalized for two or more days in the previous 90 days (27.7% versus 52.7%; $P = 0.007$). Overall antimicrobial use in the 90 days before the collection of samples for culture was not significantly different between the groups, but the difference in the rate of use of vancomycin was significant, with 27.0% of patients in the weak biofilm group but only 2.1% of patients in the strong biofilm group receiving vancomycin ($P = 0.001$). There were fewer patients on hemodialysis in the strong biofilm-producing group (0% versus 13.5%; $P = 0.006$). Patients in the strong biofilm group had a lower number of cases of bacteremia (4.3% versus 17.6%; $P = 0.03$) and pneumonia (10.6% versus 25.7%; $P = 0.04$) in the year prior to the date of collection of the MRSA isolate tested for biofilm production (referred to here as the index date). Patients in the strong biofilm group tended to present in the outpatient setting at the time that the sample for culture was obtained (referred to here as the index culture) (51.1% versus 32.4%; $P = 0.04$) (Table 1).

Alpha-toxin was produced by 79.3% ($n = 96$) of the isolates overall (74.5% in the strong biofilm group versus 82.4% in the weak biofilm group; $P = 0.29$). Beta-toxin production was less common, with 69.4% ($n = 84$) of isolates producing beta-toxin (59.6% in the strong biofilm group versus 75.7% in the weak biofilm group; $P = 0.06$). The presence of heteroresistant vancomycin-intermediate *S. aureus* (hVISA) was rare among strong biofilm- and weak biofilm-producing isolates (8.5% versus 4.4%, respectively; $P = 0.44$). The proportions of isolates with *agr* dysfunction (61.7% versus 43.2%

TABLE 1 Baseline characteristics[a]

Characteristic	Values for patients with: Strong biofilm producers ($n = 47$)	Weak biofilm producers ($n = 74$)	*P* value
Demographic characteristics			
Mean ± SD age (yr)	67.8 ± 13.5	68.1 ± 12.8	0.90
No. (%) of male patients	44 (93.6)	72 (97.3)	0.37
No. (%) of white patients	44 (93.6)	59 (79.7)	0.04
No. (%) of patients whose residence was home	39 (83.0)	51 (68.9)	0.08
Mean ± SD wt (kg)	89.9 ± 23.0	84.4 ± 21.0	0.18
Mean ± SD BMI	29.4 ± 7.9	27.1 ± 6.4	0.08
Median (Q1–Q3) SCr (mg/dl)	0.9 (0.8–1.1)	1.3 (0.9–2.2)	0.001
Median (Q1–Q3) CL_{CR} (ml/min)	92.6 (67.6–117.6)	58.4 (31.7–89.2)	0.001
Median (Q1–Q3) Charlson comorbidity index	5.0 (3– 8)	5.0 (3–8)	0.91
No. (%) of patients with the following comorbidities:			
i.v. 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
No. (%) of patients with the following smoking status:			0.80
Nonsmoker	23 (48.9)	32 (43.2)	
Smoker	14 (29.8)	23 (31.1)	
Unknown	10 (21.3)	19 (25.7)	
No. (%) of patients with the following 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)	
Median (Q1–Q3) no. of foreign materials/devices	0 (0–1)	0.5 (0–1)	0.01
Patient history characteristics			
No. (%) of patients with previous hospitalization of ≥2 days[b]	13 (27.7)	39 (52.7)	0.007
No. (%) of patients with previous surgery[b]	13 (27.7)	18 (24.3)	0.68
No. (%) of patients receiving the following medications[b]:			
Chemotherapy/immunosuppressants	5 (10.6)	2 (2.7)	0.11
Chronic corticosteroids[c]	6 (12.8)	5 (6.8)	0.33
NSAID	19 (40.4)	35 (47.3)	0.46
Gastric acid suppressor[d]	21 (44.7)	44 (59.5)	0.11
HMG-CoA reductase inhibitor	21 (44.7)	28 (37.8)	0.45
No. (%) of patients receiving the following antimicrobials[b]:	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
Median (Q1–Q3) no. of antibiotics[b]	1 (0–2)	1 (0–2)	0.08
No. (%) of patients with the following infections[e]:			
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
No. (%) of patients with ≥1 previous *S. aureus* infection[e]	12 (25.5)	21 (28.4)	0.73
No. (%) of patients with MRSA infection	11 (23.4)	18 (24.3)	0.91

(Continued on next page)

TABLE 1 (Continued)

Characteristic	Values for patients with: Strong biofilm producers ($n = 47$)	Weak biofilm producers ($n = 74$)	*P* value
No. (%) of patients with the following source of previous *S. aureus* infection[f]:			
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
No. (%) of patients in whom the index isolate was from the same site as previous *S. aureus* isolate	9 (19.2)	12 (16.2)	0.68
No. (%) of patients with previous polymicrobial infections	13 (27.7)	23 (31.1)	0.69
No. (%) of patients MRSA nares positive[e]	6 (12.8)	15 (20.3)	0.29
Index culture characteristics			
No. (%) of patients for whom the following site was sampled for culture:			
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
No. (%) of patients with the following 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
No. (%) of patients in whom infection was trauma associated	5 (10.6)	9 (12.2)	0.80
Characteristics at time of index culture			
No. (%) of patients in the following setting:			0.04
Inpatient	23 (48.9)	50 (67.6)	
Outpatient	24 (51.1)	24 (32.4)	
No. (%) of patients with inpatient admission in:			0.70
ICU	6 (26.1)	11 (22.0)	
Non-ICU	17 (73.9)	39 (78.0)	
Median (Q1–Q3) length of stay (days)	14.0 (4.0–28.0)	12.5 (7.0–20.0)	0.47
No. (%) of patients with surgery/procedure during admission	13 (27.7)	32 (43.2)	0.08
Median (Q1–Q3) no. of hospital days prior to index culture	0 (0–3)	0 (0–2)	0.85
No. (%) of patients MRSA nares positive	10 (21.3)	26 (35.1)	0.10
No. (%) of patients with:			
Urinary Foley catheter	18 (38.3)	27 (36.5)	0.84
i.v. catheter for >48 h	6 (12.8)	20 (27.0)	0.06
Mechanical ventilation	3 (6.4)	7 (9.5)	0.74
No. (%) of patients undergoing dialysis	0	10 (13.5)	0.006

[a]A strong biofilm was identified by an OD of ≥2.0, and a weak biofilm was identified by an OD of ≤1.0. Abbreviations: BMI, body mass index; SCr, serum creatinine concentration; CL_{CR}, creatinine clearance (determined by the Cockcroft-Gault equation); NSAID, nonsteroidal anti-inflammatory drug; HMG-CoA, β-hydroxy β-methylglutaryl coenzyme A; cSSTI, complicated skin and soft tissue infection; ICU, intensive care unit; i.v., intravenous.
[b]In the previous 90 days.
[c]Prednisone at 20 mg every day or equivalent for ≥14 days.
[d]Proton pump inhibitor or H_2 antagonists.
[e]In the previous 1 year.
[f]One or more previous infection sources.

for strong biofilm- and weak biofilm-producing isolates, respectively; $P = 0.05$) and pigmentation (76.6% versus 54.1% for strong biofilm- and weak biofilm-producing isolates, respectively; $P = 0.01$) were significantly higher in the strong biofilm group. The distribution of vancomycin MICs was similar among both groups. MRSA isolates represented seven multilocus sequence typing (MLST) clonal complexes (CC); the most common were CC5 (63.6%) and CC8 (22.3%). Significantly lower proportions of strong biofilm-producing isolates were MLST CC5 (48.9% versus 73.0% for weak biofilm-producing isolates; $P = 0.007$) and significantly higher proportions were CC8 (34.0% versus 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%),

TABLE 2 Phenotypic and genotypic characteristics[a]

Characteristic	No. (%) of patients with: Strong biofilm producers ($n = 47$)	Weak biofilm producers ($n = 74$)	*P* value
Phenotypic characteristics			
Alpha-toxin production	35 (74.5)	61 (82.4)	0.29
Beta-toxin production	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 characteristics			
MLST CC			
CC5	23 (48.9)	54 (73.0)	0.007
CC8	16 (34.0)	11 (14.9)	0.01
Other[b]	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[c]	16 (34.0)	22 (29.7)	0.62

[a]A strong biofilm was identified by an OD of ≥2.0, and a weak biofilm was identified by an OD of ≤1.0. Abbreviations: *agr*, accessory gene regulator; hVISA, heteroresistant vancomycin-intermediate *S. aureus*; MLST CC, multi-locus sequence typing clonal complex.

[b]CC1, CC4, CC20, CC30, CC45, and unable to obtain genotypic characteristics (11 isolates).

[c]t004, t010, t018, t062, t064, t067, t088, t1340, t189, t1904, t2032, t242, t2666, t334, t548, t681, t693, t985, and unable to obtain genotypic characteristics (11 isolates).

and t1094 (5.8%). Significantly more *spa* type t008 isolates (25.5% versus 8.1%; $P = 0.01$) and significantly fewer *spa* type t895 isolates (2.1% versus 24.3%; $P = 0.001$) were found among the strong biofilm-producing isolates (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 odds ratio [OR], 5.43; 95% confidence interval [CI], 1.69 to 17.4). Patients in the strong biofilm group were 64% less likely to die within 90 days (adjusted OR, 0.36; 95% CI, 0.12 to 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 who used immunosuppressants within 90 days of the time of the index culture had a 33.6 times higher odds of having a strong biofilm-producing MRSA isolate (adjusted OR, 33.6; 95% CI, 1.68 to 673). Patients harboring isolates from t008 (adjusted OR, 4.54; 95% CI, 1.21 to 17.1) also had an increased risk of having a strong biofilm-producing MRSA isolate. Further, patients with isolates that were of t895 (adjusted OR, 0.02; 95% CI, <0.001 to 0.47) or that produced beta-toxin were less likely to produce strong biofilms (adjusted OR 0.31; 95% CI, 0.11 to 0.89). Patients who had an increased serum creatinine concentration (adjusted OR, 0.33; 95% CI, 0.15 to 0.72) or who had received vancomycin in the previous 90 days (adjusted OR, 0.03; 95% CI, 0.002 to 0.39) were less likely to produce strong biofilms (Table 4).

DISCUSSION

This study demonstrated that strong biofilm formation among clinical MRSA isolates is associated with increased readmission at 90 days and a trend toward decreased 90-day mortality. Strong biofilm formation was also associated with the MRSA lineage,

TABLE 3 Clinical outcomes[g]

Outcome	No. of events/no. of patients (%)		*P* value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
	Strong biofilm producers	Weak biofilm producers			
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]

[a]Adjusted for hospitalization during previous 90 days for >2 days and admission type (inpatient or outpatient setting).
[b]Adjusted for hospitalization during previous 90 days for >2 days and infection with confirmed bacteremia at the time that the index sample was cultured.
[c]Adjusted for hospitalization during previous 90 days for >2 days, MLST CC5, serum creatinine concentration, and infection with confirmed pneumonia at the time that the index sample was cultured.
[d]Adjusted for pigmentation.
[e]Adjusted for MLST CC5 and pigmentation.
[f]Adjusted for hospitalization during previous 90 days for >2 days.
[g]Abbreviations: OR, odds ratio; CI, confidence interval; ICU, intensive care unit; BMI, body mass index.

agr dysfunction, pigmentation, and several patient factors, including the serum creatinine concentration, the patient's race, and the use of immunosuppressants.

Biofilm formation has previously been 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, the patients included were primarily male and members of military services (however, they were younger than the veterans in our study), but whereas our study was only of MRSA isolates, the previous study included multiple types of bacterial cultures and found a 5-fold increased association of MRSA among the biofilm-positive group (OR, 5.09; 95% CI, 1.12 to 23.1). Overall mortality with initial infection was 16% versus 5% in the biofilm- versus non-biofilm-forming groups, respectively ($P = 0.01$), with an attributable mortality of 7% (3). Unfortunately, it is difficult to tell how many of these cases of mortality in the study were due to biofilm-forming versus non-biofilm-forming MRSA, 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 the clonal complex of the isolate and biofilm formation, which has been found in other studies (12–14). This may be due to the 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, more weak biofilm-forming isolates were CC5, which is traditionally hospital associated, and were more often associated with hospitalization within the previous 90 days, dialysis, bacteremia,

TABLE 4 Predictors of strong biofilm-producing MRSA

Variable	OR (95% CI)[a]
Beta-toxin production	0.31 (0.11–0.89)
Chemotherapy or immunosuppressant use in previous 90 days	33.6 (1.68–673)
Serum creatinine concn (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 use in previous 90 days	0.03 (0.002–0.39)

[a]Abbreviations: OR, odds ratio; CI, confidence interval.

and pneumonia within the previous year, and treatment with vancomycin. Although the difference was not statistically significant, weak biofilm-forming isolates were associated with 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 previously been associated with strong biofilm production, as well as community-acquired skin infections and colonization (12, 15). 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 *spa* types t002 and t895 are related to CC5, *spa* type t002 was not related to biofilm formation. We found that *spa* type t008 was predictive for the strong biofilm phenotype, while significantly more weak biofilm-producing isolates were *spa* type t895. At least for this subset of isolates, the *spa* type served as a better predictor of biofilm formation than the MLST CC, potentially due to the greater degree of resolution in *spa* typing. This finding is consistent with the findings of previous studies evaluating genotypically different clones of MRSA in the production of a biofilm (6, 7).

Previously published data suggest that *agr* dysfunction is associated with biofilm formation in *S. aureus* (5, 16–18). This is in line with our own data, which demonstrated that *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 formation, depending on whether the biofilm is formed *in vivo* or *in vitro* (19). *In vitro* biofilm formation may yield a relationship with *agr* function different from that for *in vivo* biofilm formation, since there is no relationship to the host response. It is suggested that the host response and the *agr*-dependent virulence factors secreted *in vivo* regulate biofilm formation (19). Previous studies have also suggested that *agr* dysfunction is associated with the development of heteroresistant vancomycin-intermediate susceptibility; however, because our overall numbers of hVISA isolates were low, we could not confirm this finding (20, 21). Beta-toxin production 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 to 0.89). Although there are limited data on the connection between beta-toxin production and biofilm formation, in previous studies, beta-toxin production was associated with skin colonization, and colonization was associated with a weak biofilm phenotype, consistent with our findings (22, 23). Alpha-toxin production has also been associated with biofilm formation, (24, 25); however, we did not quantify how much alpha-toxin that these isolates produced in this study, which may have correlated better with 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 biofilm formation. 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 the data entered into the patient electronic medical record. To minimize selection bias, the investigator collecting clinical data was blind to the biofilm formation status of each isolate. Biofilm formation was determined using a standard assay (26–30). Additionally, we utilized a negative-control isolate to ensure comparability between results. By removing the moderate biofilm production category, we may have limited our power in the number of isolates, but the isolates tested had the most different biofilm formation 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, patient demographics, and patient clinical characteristics. Patients with a strong

biofilm-forming MRSA isolate were 5 times more likely to be admitted or readmitted within 90 days and tended to have decreased mortality at 90 days.

MATERIALS AND METHODS

Study design, population, and bacterial isolates. A retrospective cohort study was conducted among a sample of inpatients and outpatients from whom samples for culture for MRSA were collected from any site at the Providence, RI, Veterans Affairs Medical Center (PVAMC), a 119-bed federal hospital, from May 2004 to October 2013. Nares swab specimens collected for infection control surveillance purposes were excluded. Duplicate isolates that had the same multilocus sequence typing (MLST) clonal complexes (CC) and that were collected on the same date or from the same 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 PVAMC.

Microbiological (phenotypic and genotypic) data. (i) Biofilm formation assay. Biofilm formation was determined using a modified Christensen method as previously described by our group (26, 27, 31–33). *Staphylococcus epidermidis* ATCC 35984 and methicillin-susceptible *Staphylococcus aureus* ATCC 35556 were used as positive controls. An isogenic accumulation-negative mutant of ATCC 35984, M7, was used as a negative control (28, 29, 34). After incubation, planktonic bacteria were removed by rinsing each well three times with sterile Millipore water. The plates were dried overnight and then stained with 0.1% crystal violet for 15 min. Adherent stain was resolubilized with 33% glacial acetic acid for 1 h before measuring the optical density (OD) at 570 nm on a spectrophotometer (model 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) was subtracted from the OD of wells containing isolates to remove background readings. The mean OD was calculated for each isolate, using at least four replicates (34, 35). We used the degree of biofilm production, where strong biofilm production was an OD of $\geq$2.0, moderate biofilm production was an OD of $<$2.0 but $>$1.0, and weak biofilm production was an OD of $\leq$1.0, as previously described (36). For this study, we excluded moderate biofilm-producing isolates.

(ii) 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 24 h of incubation at 37°C (20). The plates were then refrigerated at 4°C for 24 h to evaluate beta-toxin production, indicated by green-brown hemolysis.

(iii) Determination of *agr* operon function. The function of the *agr* operon was measured qualitatively by determination of delta-toxin production (20, 37). 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 incubating the bacteria at 37°C for 24 h. The presence of synergistic hemolysis between the streak and the beta-lysin disk indicated the production of delta-hemolysis and, therefore, a functional *agr* locus (20, 37). 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 (37). Reference strains RN4420 and RN6607 were used as negative and positive controls for delta-toxin production, respectively.

(iv) hVISA presence. Screening for heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) was conducted using Etest glycopeptide resistance detection (GRD) strips (bioMérieux, Durham, NC) (38). Testing was conducted according to the 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 h after incubation. Standard vancomycin Etests were also conducted, according to the manufacturer's instructions, on Mueller-Hinton agar for 24 h. Heteroresistance was defined as a vancomycin or teicoplanin MIC of $\geq$8 μg/ml on the GRD Etest plus a standard vancomycin MIC of $<$4 μg/ml. Quality control of susceptibility testing was performed with reference strain ATCC 700698 (Mu3, hVISA).

(v) Pigmentation. Golden pigmentation was evaluated after overnight growth on tryptic soy agar at 37°C (39, 40). Each strain was compared to a reference white strain of *S. epidermidis* ATCC 35984 and categorized as pigmented or nonpigmented. *S. aureus* ATCC 35556 served as a pigmented control. A selection of 60 strains was categorized independently by a second reviewer, with 98.3% agreement between reviewers being obtained.

(vi) Genotyping. The staphylococcal protein A (*spa*) genotype was determined by PCR as previously described with primers 1095F and 1517R (41). Gene sequences were determined using Sanger sequencing with the forward primer only, unless the reverse primer was necessary for sequence clarification. The *spa* type was mapped to a 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 a literature search.

Patient data. Patient data were 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 the index culture (hospitalization of $>$72 h 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, nonsteroidal anti-inflammatory drugs [NSAID], β-hydroxy β-methylglutaryl coenzyme A reductase inhibitors [statins]) (42–46), the presence of prosthetic/foreign devices (i.e., orthopedic, cardiovascular, urinary Foley, and intravenous catheters), and infection/colonization history in the previous year.

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

The index date was defined as the date of collection of the MRSA isolate tested for biofilm production (index culture). MRSA infection was confirmed from microbiology data and the diagnosis of infection in the medical record. Readmission was defined as admission for any reason after the date of discharge from the admission in which the index culture was obtained. 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 the χ^2 or Fisher exact test for categorical variables and the *t* test or the Wilcoxon rank-sum test for continuous variables. Logistic regression models were used to quantify the effect of strong biofilms on each clinical outcome, while controlling for confounders of the exposure-outcome relationship (47). In multivariable modeling, a manual, non-computer-generated backward elimination approach was implemented. Logistic regression was also used to identify independent predictors associated with MRSA strong biofilm production (47). 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, software (SAS Institute, Cary, NC), with a two-tailed α value of 0.05 being required for statistical significance.

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