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CLINICAL THERAPEUTICS

Relationship between Vancomycin MIC and Virulence Gene Expression in Clonal Complexes of Methicillin-Susceptible *Staphylococcus aureus* Strains Isolated from Left-Sided Endocarditis

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AQ: A **ABSTRACT** Higher vancomycin MICs have been associated with more complicated courses and higher mortality rates in patients with *Staphylococcus aureus* bacteremia and infective endocarditis (IE). The aim of this study was to investigate whether the strains belonging to the cohort of 93 patients from a previously published study in which patients with strains with vancomycin MICs of $\geq$1.5 μg/ml presented higher mortality rates and systemic emboli than patients with strains with vancomycin MICs of $<$1.5 μg/ml had specific patterns of virulence factors, clonal complex (CC) types, or the ability to form biofilms. Vancomycin MICs were determined by Etest, and the isolates underwent *spa* typing to infer the CC, biofilm studies, a thrombin-induced platelet microbicidal assay, and multiplex PCR for the presence of virulence genes. We found no differences in genes encoding adhesins, toxins, or other putative virulence genes according to the vancomycin MIC group. CC30, CC34, and CC45 represented nearly half of the isolates, and there was no association with the vancomycin MIC. *agr* subgroups I and III predominated, with no association with the vancomycin MIC. Isolates with higher vancomycin MICs exhibited a poorer ability to form biofilms with and AQ: B without the presence of vancomycin (2.03 versus 2.48 [$P < 0.001$], respectively, for isolates with higher vancomycin MICs and 2.60 versus 2.87 [$P = 0.022$], respectively, for isolates with lower vancomycin MICs). In the multivariable analysis, *efb* and *V8* were risk factors for major emboli (for *efb*, adjusted odds ratio [aOR] = 7.5 and 95% confidence interval [CI] = 1.2 to 46.6 for *V8*, and aOR = 3.9 and 95% CI = 1.1 to 14.1), whereas no genotypic predictors of in-hospital mortality were found. No clear associations between genes encoding virulence factors, *agr* type, clonal complexes, mortality, and major embolic events according to vancomycin MIC group were found.

KEYWORDS *Staphylococcus aureus*, endocarditis, vancomycin MIC, virulence factors, clonal complex, biofilm, mortality, emboli, prognosis, *agr*, biofilms, infective endocarditis

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Methicillin-susceptible *Staphylococcus aureus* (MSSA) is the leading cause of infective endocarditis (IE) worldwide (1). Despite the advances in diagnosis and management, the in-hospital mortality rates from MSSA IE have remained stable at 20 to 25% over the last 3 decades (2, 3).

The impact of the high-vancomycin-MIC (HVM) phenotype (vancomycin MIC, ≥1.5 μg/ml) on the prognosis of MSSA bacteremia and IE is poorly understood. MSSA strains causing bloodstream infections are known to possess a distinct repertoire of virulence genes and to be members of clonal complexes (CC) that confer a virulence profile different from that of methicillin-resistant *S. aureus* (MRSA) strains (4).

Several studies have reported higher rates of complications and mortality in patients with MSSA bacteremia caused by strains with high vancomycin MICs than in patients with MSSA bacteremia caused by strains with low vancomycin MICs (LVM) (5, 6) and a correlation of high vancomycin MIC with *agr* dysfunction, *agr* type II polymorphisms, and other specific findings shaping a repertoire of virulence factors in these strains (7–10). In a cohort of 93 patients with left-sided MSSA IE treated with cloxacillin, our group found significantly higher rates of mortality and systemic emboli in the group with isolates with high vancomycin MICs than in the group with isolates with low vancomycin MICs (11). Nonetheless, some recent studies found no significant differences either in the *agr* subgroup and function in MSSA bacteremia and IE (12, 13) or on the outcomes of left-sided MSSA IE treated with beta-lactams (13) according to the vancomycin MIC.

The aim of this study was to investigate whether the strains belonging to the cohort of patients from our previously published study (11) presented specific patterns of virulence factors, clonal complex types, or ability to form biofilms in the presence of vancomycin according to the vancomycin MIC group.

(The data presented in this study were reported in part at the 27th European Conference on Clinical Microbiology and Infectious Diseases [ECCMID], Vienna, Austria, 22 to 25 April 2017 [14], and at the 15th International Symposium on Modern Concepts in Endocarditis and Cardiovascular Infections [ISCVID], Lausanne, Switzerland, 2 to 4 June 2019.)

RESULTS

The distribution of vancomycin MICs over the study time period, which was significantly different between the subperiod from 1995 to 2002 and the subperiod from 2003 to 2011 ($P = 0.026$), is shown in Fig. S1 in the supplemental material.

No differences in genes encoding adhesins (fibronectin-binding proteins [*fnbA*, *fnbB*], clumping factors [*clfA*, *clfB*], the collagen-binding antigen [*cna*], serine-aspartate repeat proteins for adhesion [*sdrC*, *sdrD*, *sdrE*], sialoprotein [*bbp*] and elastin-binding protein [*ebps*], and major histocompatibility complex [MHC] class II analog proteins [MAP/EAP]), toxins (exfoliative toxins [*eta*, *etb*], enterotoxins [*tst*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*], staphylococcal enterotoxins [*sea*, *seb*], Panton-Valentin leucocidin [PVL]), or other putative virulence genes (extracellular fibrinogen-binding protein [*efb*], adhesion intracellular protein A [*icaA*], chemotaxis-inhibiting protein [*chp*], and serine endopeptidase [*V8*]) were found between the MSSA isolates according to the vancomycin MIC group (~~high vancomycin MIC~~ [HVM] and ~~low vancomycin MIC~~ [LVM]). *agr* subgroups I and III predominated, with no association of the *agr* subgroup with the vancomycin MIC group being found (Table 1).

CC30, CC34, and CC45 altogether represented nearly half of the isolates (22.6%, 10.6%, and 16.3%, respectively), and there was not an association either with the vancomycin MIC group (Table 2) or with the study subperiod timelines (Fig. S2). Isolates with LVM exhibited a greater ability to form a biofilm with and without the presence of vancomycin (2.48 versus 2.03 [$P < 0.001$], respectively, for isolates with HVM and 2.87 versus 2.60 [$P = 0.022$], respectively, for isolates with LVM) (Table 2).

In the univariate analysis, neither the type of clonal complex, the biofilm formation ability, nor the virulence factors present were identified to be risk factors for in-hospital mortality, except for the presence of *efb*, which was associated with a lower mortality

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TABLE 1 Phenotypic and genotypic characteristics of methicillin-susceptible *Staphylococcus aureus* bloodstream isolates from patients with infective endocarditis according to vancomycin MIC

Gene	No. (%) of isolates for which vancomycin MIC was: <1.5 mg/ (*n* = 53)	>1.5 mg/li = 40)	*P*
Adhesins			
fnbA	53 (100)	40(100)	1
fnbB	41 (77.4)	37 (92.5)	0.085
clfA	46 (86.8)	36 (90)	0.752
clfB	45 (84.9)	35 (87.5)	0.772
cna	23 (43.4)	18 (45)	0.865
sdrC	20 (37.7)	18 (45)	0.527
sdrD	38 (71.7)	26 (65)	0.507
sdrE	22 (41.5)	19 (47.5)	0.674
bbp	29 (54.7)	26 (65)	0.395
ebpS	33 (62.3)	29 (72.5)	0.376
map and *eap*	3 (5.7)	4 (10)	0.458
Toxins			
eta	4 (7.5)	7 (17.5)	0.197
etb	0 (0)	0 (0)	1
tst	40 (75.5)	33 (82.5)	0.456
sea	42 (79.2)	29 (72.5)	0.470
seb	6 (11.3)	5 (12.5)	0.890
sec	5 (9.4)	5 (12.5)	0.740
sed	0 (0)	1 (2,5)	0.430
see	0 (0)	0 (0)	1
seg	40 (75.5)	33 (82.5)	0.456
seh	9 (17)	3 (7.5)	0.222
sei	34 (64.2)	26 (65)	1
sej	20 (37.7)	13 (32.5)	0.665
pvl	0 (0)	1 (2.5)	0.430
Other putative virulence genes			
efb	35 (66)	29 (73)	0.652
icaA	53 (100)	40 (100)	1
chp	27 (50.9)	20 (50)	1
V8	10 (18.9)	8 (20)	0.890
agr group			
agr type I	17 (32.1)	15 (37.5)	0.661
agr type II	12 (23)	9 (22.5)	1
agr type III	20 (37.7)	12 (30)	0.511
agr type IV	2 (3.8)	0 (0)	0.504
Unknown *agr* type	2 (3.8)	4 (10)	0.526

(Table 3). CC5 was significantly associated with a higher mortality, whereas CC34 and the presence of *efb* were significantly associated with a lower 1-year mortality. As regards clinically evident systemic emboli, the presence of *sei*, *efb*, and *V8* and a lower ability for biofilm formation in the presence of vancomycin were significantly associated with higher rates of emboli, whereas the ability to form a biofilm in the presence of vancomycin was associated with a reduced risk of emboli (Table 4). No genotypic predictors for in-hospital mortality were found in the multivariable analysis (Table 5). Conversely, the presence of *efb*, together with that of *V8*, was a risk factor for symptomatic major emboli (Table 6).

DISCUSSION

In our previous study published 5 years ago, we found that the rates of in-hospital and 1-year mortality were significantly higher in the HVM group of 93 cases with left-sided MSSA IE treated with beta-lactams (11). Along with this finding, we observed that patients whose isolates had HVM had significantly more systemic emboli. In the multivariate analysis, HVM and emboli were associated with higher rates of in-hospital

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TABLE 2 Clonal complexes, biofilm production, and tPMP activity of MSSA bloodstream isolates from patients with IE according to vancomycin MIC

Characteristic	Value for isolates for which vancomycin MIC was:		*P*
	<1.5 mg/liter (*n* = 53)	>1.5 mg/liter (*n* = 40)	
No. (%) of isolates of the following CC			
CC5	3 (6)	6 (15)	0.166
CC15	7 (13)	1 (3)	0.132
CC25	2 (4)	4 (10)	0.397
CC30	12 (23)	9 (23)	0.987
CC34	7 (13)	3 (8)	0.507
CC45	8 (15)	7 (18)	0.755
Other	14 (26)	10 (25)	0.877
Biofilm formation (SD)			
Without vancomycin	2.871 (0.685)	2.602 (0.417)	0.022
With vancomycin	2.482 (0.509)	2.034 (0.421)	<0.001
tPMP activity at threshold concn (mg/liter) of[a]:			
50	64.442 (18.595)	72.764 (23.918)	0.072
25	80.183 (21.271)	87.298 (20.607)	0.108
12.5	76.665 (18.593)	80.845 (18.861)	0.290

[a]tPMP, thrombin-induced platelet microbicidal protein. The values are means (SDs).

mortality. Major embolism is the direct reason for death in a remarkable proportion of IE cases, for which vegetation size and severe mitral regurgitation are the most important predictors (15, 16). The clinical trial of Kang et al. (16), comparing an early versus a conventional surgical approach for IE, was based on these predictors and led to a revision of the latest international guidelines to include the size of vegetations as a criterion for urgent surgery (17, 18). In our previous study, we did not analyze differences in the sizes of the vegetations between the HVM and LVM groups (11). Moreover, despite hypothesizing that higher mortality rates in the HVM subgroup might be related to embolic events, with a possible *agr* quorum-sensing system dysfunction being the underlying mechanism (7–10), the association was not evaluated.

In the present study, we analyzed the genotypic characteristics and the ability to form a biofilm of the 93 strains of *S. aureus* included in the previous study. The main aim was to find a genetic background that may be associated with the different phenotypes of the vancomycin MIC, because a positive finding might lead to specific therapeutic recommendations, such as early cardiac surgery. Unfortunately, our results do not clarify whether the genetic background of the strains is associated with a worse patient prognosis according to the vancomycin MIC group. CC5 was associated with higher mortality at 1 year (although at a low rate of 18%) in the univariate analysis, while in the multivariate analysis, it was found to be a risk factor for major embolic events but not for in-hospital mortality. The genes encoding extracellular fibrinogen binding protein (*efb)* and serine endopeptidase (*V8*) were associated with a higher likelihood of suffering major embolic events, whereas no genotypic features were identified to be predictors of mortality in the multivariable analysis. The rest of our findings regarding a potential impact of genes encoding adhesins, toxins, other virulences genes, clonal complexes, and *agr* types were also inconclusive.

Interestingly, the ability of HVM strains to form biofilms in the presence or in the absence of vancomycin was significantly lower than that of LVM strains. Furthermore, we found a significant association of *efb* and *V8* with embolic events. *efb* participates in *S. aureus* evasion from phagocytosis by complement, in the inhibition of platelet aggregation, and in the inhibition of the formation of platelet-leukocyte complexes (19–21). *V8* encodes a protease that is involved in the cleavage and inhibition of a wide array of proteins, including complement and fibronectin-binding proteins, among other functions (22). This may explain the higher rate of major embolisms found in the HVM group, since a poorer ability to generate stable biofilms could potentially lead to more

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TABLE 3 Univariate analysis for in-hospital and 1-year mortality

Characteristic[a]	In-hospital mortality			1-yr mortality		
	Value for:			Value for:		
	In-hospital survivors ($n = 56$)	Patients with in-hospital mortality ($n = 37$)	*P*	1-yr survivors ($n = 53$)	Patients with 1-yr mortality ($n = 40$)	*P*
No. (%) of patients with isolates expressing the following adhesins:						
fnbA	56 (100)	37 (100)		53 (100)	40 (100)	
fnbB	46 (82)	32 (87)	0.577	43 (81)	35 (88)	0.408
ebpS	39 (70)	23 (62)	0.454	37 (70)	25 (63)	0.459
No. (%) of patients with isolates expressing the following toxins:						
sei	38 (68)	22 (59)	0.407	35 (66)	25 (63)	0.724
sej	16 (29)	17 (46)	0.087	15 (28)	18 (45)	0.096
pvl	0 (0)	1 (3)	0.398	0 (0)	1 (3)	0.430
No. (%) of patients with isolates expressing the following other putative virulence genes:						
efb	43 (77)	21 (57)	0.041	41 (77)	23 (58)	0.041
icaA	56 (100)	37 (100)		53 (100)	40 (100)	
chp	29 (52)	18 (49)	0.767	29 (55)	18 (45)	0.353
V8	10 (18)	8 (22)	0.653	9 (17)	9 (23)	0.505
No. (%) of patients with isolates with the following *agr* subgroup:						
I	20 (36)	12 (32)	0.744	20 (38)	12 (30)	0.437
II	11 (20)	10 (27)	0.405	9 (17)	12 (30)	0.137
III	22 (39)	10 (27)	0.223	22 (42)	10 (25)	0.097
IV	2 (4)	0 (0)	0.516	1 (2)	1 (3)	1.000
No. (%) of patients with isolates of the following CCs:						
CC5	4 (7)	5 (14)	0.475	2 (4)	7 (18)	0.036
CC15	6 (11)	2 (5)	0.470	6 (11)	2 (5)	0.459
CC25	4 (7)	2 (5)	1.000	4 (8)	2 (5)	0.696
CC30	12 (21)	9 (24)	0.744	12 (23)	9 (23)	0.987
CC34	9 (16)	1 (3)	0.083	9 (17)	1 (3)	0.039
CC45	9 (16)	6 (16)	0.985	9 (17)	6 (15)	0.797
Other	12 (21)	12 (32)	0.235	11 (21)	13 (33)	0.200
Biofilm formation (						
Without vancomycin	2.75 (0.66)	2.76 (0.49)	0.915	2.74 (0.66)	2.78 (0.50)	0.790
With vancomycin	2.30 (0.50)	2.27 (0.55)	0.820	2.30 (0.52)	2.27 (0.53)	0.776
tPMP activity at threshold concn (mg/liter) of[b]:						
50	69.34 (21.7)	66.03 (20.9)	0.467	68.7 (22.1)	67.1 (20.5)	0.723
25	84.59 (23.1)	81.20 (17.9)	0.453	83.7 (23.1)	82.6 (18.6)	0.806
12.5	78.92 (18.6)	77.77 (19.1)	0.772	78.4 (18.6)	78.6 (19.1)	0.956

[a]The following is the complete list of adhesins and toxins assessed: *fnbA*, *fnbB*, *clfA*, *clfB*, *cna*, *sdrC*, *sdrD*, *sdrE*, *bbp*, *ebpS*, *map* (*eap*), *eta*, *etb*, *tst*, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *she*, *sei*, *sej*, *pvl*, *and hlg*.
[b]tPMP, thrombin-induced platelet microbicidal protein. The values are means (SDs).

friable vegetations with subsequent dislodgement from the valve surface and major systemic emboli. Nonetheless, we have not found distinct genetic features of HVM strains explaining their lower ability to form a biofilm. Moreover, we did not find a significant association between the expression of *efb* and *V8* and the formation of a biofilm (with and without vancomycin; data not shown). Despite an association between biofilm formation and major emboli in the univariate analysis, this finding could not be confirmed in the multivariate analysis, likely due to a lack of potency. Thus, we have not found a robust association between outcomes, e.g., a phenotypic characteristic, such as biofilm formation, and genotypic features.

In a recent large prospective cohort study including 212 patients, Fernández-

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TABLE 4 Univariate analysis for major symptomatic embolic events

Characteristic[a]	Value for patients with: No embolic events (*n* = 74)	Embolic events (*n* = 19)	*P*
No. (%) of patients with isolates expressing the following adhesins:			
fnbA	74 (100)	19 (100)	
fnbB	63 (85)	15 (79)	0.499
ebpS	46 (62)	16 (84)	0.069
No. (%) of patients with isolates expressing the following toxins:			
sei	44 (60)	16 (84)	0.044
sej	27 (37)	6 (32)	0.792
pvl	0 (0)	1 (5)	0.204
No. (%) of patients with isolates expressing the following other putative virulence genes:			
efb	47 (64)	17 (90)	0.029
icaA	74 (100)	19 (100)	
chp	35 (47)	12 (63)	0.217
V8	11 (15)	7 (37)	0.048
No. (%) of patients with isolates with the following *agr* subgroup:			
I	25 (34)	7 (37)	0.802
II	16 (22)	5 (26)	0.759
III	26 (35)	6 (32)	0.771
IV	1 (1)	1 (5)	0.369
No. (%) of patients with isolates of the following CCs:			
CC5	6 (8)	3 (16)	0.382
CC15	8 (11)	0 (0)	0.200
CC25	4 (5)	2 (11)	0.598
CC30	19 (26)	2 (11)	0.224
CC34	7 (10)	3 (16)	0.681
CC45	13 (18)	2 (11)	0.520
Other	17 (23)	7 (37)	0.246
Biofilm formation ( ):			
Without vancomycin	2.75 (0.58)	2.79 (0.66)	0.811
With vancomycin	2.35 (0.53)	2.06 (0.41)	0.028
tPMP activity at threshold concn (mg/liter) of[b]:			
50	67.3 (21.1)	70.8 (22.3)	0.549
25	82.5 (21.6)	86.1 (19.7)	0.508
12.5	78.4 (19.0)	78.8 (18.2)	0.930

[a]The complete list of adhesins and toxins assessed is as follows: *fnbA, fnbB, clfA, clfB, cna, sdrC, sdrD, sdrE, bbp, ebpS, map* (*eap*), *eta, etb, tst, sea, seb, sec, sed, see, seg, she, sei, sej, pvl, and hlg.*

[b]tPMP, thrombin-induced platelet microbicidal protein. The values are means (SDs).

Hidalgo et al. analyzed whether an HVM impacted the prognosis of *S. aureus* IE (23). They found no significant association between HVM and in-hospital mortality either in MRSA IE cases or in MSSA IE cases (when cases of left- and right-sided IE were pooled). Moreover, *agr* dysfunction and CC5 were also not associated with higher in-hospital mortality, whereas symptomatic central nervous system (CNS) but not peripheral emboli were associated with higher mortality rates. In the univariate analysis, *agr* type III and CC30 were significantly associated with lower mortality rates and CC8 was significantly associated with higher mortality rates in patients with MSSA IE (23).

In another study by the same group, Viedma et al. recently showed that *in vitro* exposure to subinhibitory concentrations of oxacillin of MRSA and MSSA strains from patients with endovascular infections induced changes in *agr* expression that varied depending on the CC, which could potentially impact the virulence of the strains and, therefore, the course of the infection (24). Notably, no correlation between the oxacillin MIC and the vancomycin MIC was found in our previous study (11).

After studying strains from patients with left-sided MSSA IE from the International

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TABLE 5 Multivariate analysis of risk factors for in-hospital mortality[a]

	Univariate analysis		Multivariable analysis	
Characteristic	OR	95% CI	OR	95% CI
Age	1.006	0.994–1.018		
Expression of:				
bbp	0.486	0.208–1.138		
sej	2.125	0.892–5.064	2.210	0.889–5.495
efb	0.397	0.162–0.975	0.428	0.126–1.410
CC5	2.031	0.508–8.127		
CC34	0.145	0.018–1.198	0.177	0.021–1.504

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

Collaboration on Endocarditis, we did not find significant differences in mortality or in systemic embolic events between the HVM and LVM groups, which, as in the present study, had similar distributions of virulence genes and clonal lineages (13). Neither specific *agr* types nor specific CCs were associated with higher mortality, whereas the presence of *sei* was. The study findings were, in any case, constrained by a poor statistical potency due to a small sample size (62 cases).

Although they are likely not definitive, the results of recent studies, along with those of the present one, suggest that the association between HVM and worse outcomes found in our previous study may not represent a direct association but a surrogate marker of other complex mechanisms. In light of the interesting results provided by Viedma et al. (24), one plausible explanation may be that in our previous study, plasma and/or vegetation drug levels during treatment may have induced a variety of modifications in *agr* expression, leading to worse outcomes in the HVM group. Another question requiring further research is whether the changes in the virulence of MSSA strains causing IE affect their ability to generate larger and more friable vegetations in relation to a poorer ability to form biofilms and whether this phenomenon may be related, or not, to exposure to subinhibitory concentrations of cloxacillin and, eventually, to higher rates of major emboli and death. Any of those scenarios would entail therapeutic consequences, such as optimizing the pharmacokinetic/pharmacodynamic properties of cloxacillin (e.g., by the use of continuous infusion and larger loading doses), avoiding the use of cloxacillin in a subset of MSSA IE patients bearing specific features to be defined, or anticipating cardiac surgery. With the available evidence, we cannot use the vancomycin MIC as a predictive variable to develop a selective management strategy for MSSA IE. As extensively shown by and discussed in earlier literature, the absence of a correlation between genetic markers of bacterial virulence and either clinical expression or outcome prediction is not rare. This does not entail either the genetic or the phenotypic determinants of bacterial infections being involved in a particular outcome but, rather, suggests that researchers should be able to

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TABLE 6 Multivariate analysis of risk factors for major symptomatic emboli[a]

	Univariate analysis		Multivariable analysis	
Characteristic	OR	95% CI	aOR	95% CI
Presence of:				
efb	4.883	1.047–22.770	7.504	1.207–46.640
ebpS	3.246	0.868–12.147		
V8	3.341	1.078–10.352	3.951	1.103–14.146
sei	3.636	0.974–13.579		
CC5	2.125	0.479–9.420	6.657	0.953–46.493
CC34	1.795	0.417–7.715		
Biofilm formation with vancomycin	0.273	0.084–0.889	0.274[b]	0.071–1.049

[a]OR, odds ratio; aOR, odds adjusted ratio; CI, confidence interval.
[b]Per 1-unit increase of biofilm formation ability.

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approach nonlinear effects in the clinical setting by also considering epigenetic stimuli. Therefore, further studies exploring both the biofilm formation of MSSA strains causing bloodstream infections and outcomes are needed.

This study is constrained by several limitations. First, the strains analyzed were collected over a long period, so the results are subject to a historical bias and to the risk of a storage effect on the vancomycin MICs. Second, all IE cases came from a single center and almost 8 years have passed since the last patient was included, therefore leading to constraints in external validity. Third, as the study of Viedma et al. showing the changes induced in *agr* expression with different oxacillin subinhibitory concentrations (24) had not been published at the time of our experiment stage, we were unable to assess this association in our collection. Last, we may expect a type II error in some of the subanalyses due to the small sample size.

In conclusion, we did not find that a distinct repertoire of virulence genes, clonal lineages, or ability to form a biofilm explained the worse prognosis of patients with IE caused by MSSA strains with a high vancomycin MIC treated with cloxacillin.

MATERIALS AND METHODS

Subjects and isolates. The cohort included 93 *S. aureus* isolates (53 strains with vancomycin MICs of <1.5 μg/ml and 40 strains with vancomycin MICs of ≥ 1.5 μg/ml) collected between 1995 and 2011 from patients with definite MSSA left-sided endocarditis. Vancomycin MICs were determined by Etest in duplicate. The demographic, clinical, echocardiographic, and outcome variables of the 93 patients with left-sided MSSA IE whose strains were collected for the present study were defined and are reported elsewhere (11). Notably, patients with MSSA IE caused by strains with HVM and strains with LVM did not significantly differ in any important baseline or clinical characteristics (e.g., type of acquisition, valve involvement, native or prosthetic IE, periannular complications, etc.), with the exception of systemic emboli, which were more frequent in the former group, which also had a higher probability of in-hospital and 1-year mortality. Besides the HVM, other risk factors for mortality were septic complicated endocarditis (in which the patients presented with either severe sepsis or septic shock at admission) and nonseptic complicated endocarditis (defined as having one or more of the conditions systemic emboli, periannular abscess, and heart or renal failure at the baseline or during the first 2 weeks of follow-up) (11). All patients admitted to the Hospital Clinic of Barcelona included in the prior clinical study on which the present work is based provided either oral or written informed consent for the inclusion and analysis of their data in the Hospital Clinic Endocarditis Prospective Database (25). The Institutional Review Board of the Hospital Clinic de Barcelona provided approval. The article does not contain any individual person's data that might lead to the identification of the patients included in the study.

AQ: K

Definitions. IE was defined according to the modified Duke criteria (26) and was considered to be left sided when no right-sided (tricuspid or pulmonary valve) vegetations were present on echocardiography, surgery, or autopsy. A strain was considered to have an HVM when the MIC was ≥ 1.5 μg/ml by Etest and an LVM when it was <1.5 μg/ml. The rest of the definitions have been previously provided in detail (3).

Multiplex PCR. Genomic DNA was prepared as previously described (4). Bacterial determinants, including adhesins, toxins, *agr* group I to IV, and other genes, were screened by multiplex PCR ~~(180 PCR)~~ as previously described (4). All negative calls in the multiplex PCR were confirmed by uniplex PCR.

AQ: L

***spa* typing.** *spa* typing was performed as previously described (4, 9). PCR oligonucleotide primers for *spa* were previously described (9). Samples were sequenced at the Duke University sequencing laboratory. For *spa* typing, eGenomics software was used to scan the primary sequence in order to help to identify the orders and names of each repeat. The *spa* type number is representative of the repeat organization. Clonal complexes for the isolates were identified via repeat pattern recognition from the existing *spa* type and CC database, previously confirmed via multilocus sequence typing (MLST). Isolates whose *spa* type did not map to a known CC underwent MLST. ~~For MLST, the sequence chromatograms for unique alleles were deposited in the MLST database (http://www.mlst.net). The alleles at seven loci (*arcC, aroE, glpF, gmk, pta, tpi,* and *yqiL*) were used to identify a unique sequence type (ST). MLST allele names and STs were derived from http://www.mlst.net. CCs were assigned to groups of isolates sharing six of seven alleles by using the eBURST algorithm (http://eburst.mlst.net)~~ (27).

AQ: M

AQ: N

Biofilm formation. Biofilm formation experiments were conducted at the fLos Angele[illegible]medical Research Institute at Harbor-UCLA following the methodology presented elsewhere (28).

Thrombin-induced platelet microbicidal assay. The activity of the thrombin-induced platelet microbicidal proteins (tPMP) of MSSA isolates was tested *in vitro* as previously described (29). In brief, the assay was performed using rabbit platelets to categorize the specific tPMP susceptibility thresholds of the MSSA isolates, using an inoculum of 10^3 CFU. For each isolate, tPMP was expressed as the mean $\pm$ standard deviation (SD) of the findings from two independent assays performed on separate days with the following thresholds: 12.5, 25, and 50 mg/liter.

Statistical analysis. Categorical variables were summarized as percentages, and continuous variables were summarized as means and standard deviations. Categorical variables were compared using the chi-square test (or Fisher's exact test, where necessary). Continuous variables were compared using the Kruskal-Wallis test. We did not include epidemiological, clinical, or echocardiographic variables

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from the patients harboring isolates with either HVM or LVM in the analyses since we already knew the effect of these variables on mortality and major emboli from our previous study (11). Rather, we pursued to assess whether the analyzed genotypic and phenotypic variables of the strains collected from patients with IE from the HVM and LVM groups were indeed associated with outcomes equivalent to those found in our previous study. For the analysis of risk factors of mortality and major symptomatic emboli, a logistic regression model that included variables with P values of <0.30 in the univariate analysis was used. A two-sided P value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA).

Data availability. The data sets generated and/or analyzed during the current study are not publicly available for confidentiality reasons related to ongoing research but are available from the corresponding author on reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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V.G.F. reports the following potential conflicts of interest: chair of the Scientific Advisory Board for Merck V710; paid consultant for Pfizer, Novartis, Galderma, Novadigm, Durata, Debiopharm, Genentech, Achaogen, Affinium, The Medicines Co., Cerexa, Tetraphase, Trius, MedImmune, Bayer, Theravance, Cubist, Basilea, and Affinergy; grants pending from MedImmune, Actavis/Forest/Cerexa, Pfizer, Merck/Cubist, Advanced Liquid Logics, Theravance, Novartis, Medical Surfaces, and Locus Biotechnology; royalties from UpToDate; personal fees for development or presentation of educational presentations (Green Cross, Cubist, Cerexa, Durata, Theravance) outside the submitted work; and a patent pending related to sepsis diagnostics. J.M.M. has received consulting honoraria and/or research grants from Angelini, Contrafect, Genentech, Gilead, Jansen, Medtronic, MSD, Novartis, Gilead Sciences, and ViiV Healthcare. None of the other authors has a conflict to declare.

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AUTHOR QUERIES

Below are queries from the copy editor indicating specific areas of concern. Please respond in-line in the main text above, either by marking a change or indicating "ok."

1

AQau—Please make certain that all authors' names are spelled correctly, and confirm the given-names and surnames are identified properly by the colors (this is important for how the names are indexed).
■= Given-Name, ■= Surname

AQabbr—Please check any added introductions of abbreviations and correct them if necessary.

AQfund—The table below includes funding information that you provided on the submission f[illegible]n when you submitted the manuscript. This funding information will not appear in the article, but it will be provided to CrossRef and made publicly available. Please check it carefully for accuracy and mark any necessary corrections. If you would like statements acknowledging financial support to be published in the article itself, please make sure that they appear in the Acknowledgments section. Statements in Acknowledgments will have no bearing on funding data deposited with CrossRef and vice versa.

Funder	Grant(s)	Author(s)	Funder ID
HHS | National Institutes of Health (NIH)	R01-AI068804	Vance G. Fowler	https://doi.org/10.13039/100000002
HHS | National Institutes of Health (NIH)	K24-AI093969	Vance G. Fowler	https://doi.org/10.13039/100000002
MEC | Instituto de Salud Carlos III (ISCIII)	PI14/00603	José M. Miró	https://doi.org/10.13039/501100004587
Fundació la Marató de TV3 (TV3 Marathon Foundation)	20152610	José M. Miró	https://doi.org/10.13039/100008666

AQA—Au: (i) Is the title accurate as edited? (ii) Each affiliation is given a separate letter per ASM style, so old affiliation e is now affiliations e and f. Please check the affiliation relettering throughout.

AQB—Au: (i) Are the data in parentheses (i.e., 2.03, 2.48, 2.60, and 2.87) accurate as explained? (ii) Throughout the article, please provide units for the biofilm formation data or otherwise indicate to what the data refer.

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2

AQC—To ensure sequential order, references have been renumbered in the text and References as a new reference 14 was added per ASM style to cite the abstract from the 27th ECCMID cited in the paragraph at the end of the introduction and to fix the double references 16 and 17 that appeared in the manuscript (many thanks for your prompt help in fixing that). Please check and correct the renumbering if necessary. If any reference should be deleted from the References list, please mark "Reference deleted" in the margin next to that entry; do not renumber subsequent references.

AQD—Au: In the sentence beginning " MSSA strains causing bloodstream infections are known to possess," if insertion of "different from that of methicillin-resistant *S. aureus* (MRSA) strains" to specify that from which the "virulence profile" is different is not accurate, please explain "different."

AQE—Au: In the sentence beginning "Several studies have reported higher rates of complications and mortality," if the changes made to complete the comparison indicated by "higher" and to specify "correlation" are not accurate, please explain as appropriate for complete and parallel comparison and to explain the things that correlate. Please also check the changes in the next sentence made to complete the comparison indicated by "higher."

AQF—Au: Slashes are not used with bacterial genotypes per ASM style, unless complementation is meant. If the replacement of the slash with "and" is not accurate, please explain as appropriate in the proofs.

AQG—Au: Dashes are not used as placeholders in tables unless they are defined in a footnote, ASM style.

AQH—Au: If OR and 95% CI are not accurate subheads in Table 5, please provide the correct heads to identify the data presented in each column.

AQI—Au: In footnote *a* of Table 6, if the change of "Per 1 increase" to "Per 1-unit increase" is not accurate, please explain "Per 1" as appropriate in the proofs.

AQJ—Au: In the sentence beginning "In light of the interesting results provided by Viedma et al.," if the citation of reference 24 to specify "Viedma et al." is not accurate, please provide the correct reference number.

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3

AQK—Au: The "Ethics Approval" section that appeared in Acknowledgments in the manuscript was incorporated into Materials and Methods per ASM style. Some of the same information appeared in both locations in the manuscript, so the text was edited to delete repetition. Please check the information beginning in the sentence "All patients" and to the end of the paragraph and make any necessary corrections in the proofs.

AQL—Au: Is "180 PCR" common terminology (that is, will all readers know what "180 PCR" refers to), or can the "180" be explained more fully?

AQM—Au: .com URLs are not permitted per ASM style.

AQN—Au: The URLs http://www.mlst.net and http://eburst.mlst.net take the user to the website for "Trust Pharmacy." Please check that the URLs provided are correct.

AQO—Au: Please provide the surnames and initials of all authors of the abstract cited in reference 14.