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Influence of vancomycin minimum inhibitory concentration on the outcome of methicillin-susceptible *Staphylococcus aureus* left-sided infective endocarditis treated with antistaphylococcal β -lactam antibiotics: a prospective cohort study by the International Collaboration on Endocarditis

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Title: Influence of Vancomycin Minimum Inhibitory Concentration on the Outcome of Methicillin-Susceptible *Staphylococcus aureus* Left-Sided Infective Endocarditis Treated with Anti-staphylococcal Beta-Lactam Antibiotics; a Prospective Cohort Study by the International Collaboration on Endocarditis.

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14 35 **Keypoints:** In this international cohort of patients with left-sided MSSA endocarditis
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16 36 treated with anti-staphylococcal beta-lactam antibiotics, vancomycin MIC phenotype was
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18 37 not associated with patient demographics, clinical outcome, or virulence gene repertoire.
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57 **Abstract**

58 **Objectives:** A recent study showed a poorer prognosis in left-sided MSSA endocarditis
59 treated with cloxacillin when the vancomycin MIC was ≥ 1.5 mg/L. We aimed to validate
60 these results using the International Collaboration on Endocarditis cohort and to analyze
61 whether specific genetic characteristics were associated with a high vancomycin MIC
62 (≥ 1.5 mg/L) phenotype.

63 **Methods:** All patients with left-sided MSSA IE treated with anti-staphylococcal beta-
64 lactam antibiotics between 2000 and 2006 with available isolates were included.
65 Vancomycin MIC (VAN MIC) was determined by E-test as either *high* (≥ 1.5 mg/L) or *low*
66 (< 1.5 mg/L). Isolates underwent *spa* typing to infer clonal complexes (CC) and multiplex
67 polymerase chain reaction for identifying virulence genes. Univariate analysis was
68 performed to evaluate the association between in-hospital and one-year mortality and
69 vancomycin MIC phenotype.

70 **Results:** 62 cases met the inclusion criteria. VAN MIC was *low* in 28 cases (45%) and
71 *high* in 34 cases (55%). No significant differences in patient demographic data or
72 characteristics of infection were observed between patients with IE due to *high* and *low*
73 VAN MIC isolates. Isolates with *high* and *low* VAN MIC had similar distributions of
74 virulence genes and clonal lineages. In-hospital and one-year mortality did not differ
75 significantly between the two groups (32% (9/28) vs. 27% (9/34), $P=0.780$; and 43%
76 (12/28) vs. 29% (10/34), $P=0.298$, for *low* and *high* VAN MIC, respectively).

77 **Conclusion:** In this international cohort of patients with left-sided MSSA endocarditis
78 treated with anti-staphylococcal beta-lactams, vancomycin MIC phenotype was not
79 associated with patient demographics, clinical outcome, or virulence gene repertoire.

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BACKGROUND

Methicillin-susceptible *Staphylococcus aureus* (MSSA) is the leading cause of infective endocarditis (IE) worldwide [1]. Despite the advances on diagnosis and management, the in-hospital mortality of MSSA IE has stabilized at 20-25% over the last three decades [2,3].

The relationship between vancomycin minimum inhibitory concentration (MIC) of bloodstream *S. aureus* isolates and patient outcome is unresolved. Although several studies have now shown an association between *high* MIC (≥ 1.5 mg/L) in methicillin-resistant *S. aureus* (MRSA) bloodstream isolates and a worse clinical outcome in patients with MRSA bacteremia [4], other reports have failed to confirm this observation [5]. However, no clinical studies have addressed the potential association between *high* vancomycin MIC and worse patient outcome in patients with MRSA IE. Furthermore, studies in the experimental model of IE have failed to demonstrate such associations [6].

The impact of a *high* vancomycin MIC phenotype (HVM; ≥ 1.5 mg/L) in MSSA bacteremia and IE and the relationship with *agr* dysfunction is also poorly defined. To date, several studies reported higher rates of complications and mortality in patients with MSSA bacteremia caused by strains with HVM [1,2] as well as a correlation with *agr* dysfunction and *agr* type II polymorphism [3]. Nonetheless, a more recent study did not find significant differences on *agr* subgroup and function according to the vancomycin MIC [4]. The association between HVM and significantly higher mortality was also demonstrated in a Spanish a cohort of 93 patients with MSSA IE treated with cloxacillin [5]. These clinical findings await international validation.

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107 **Objectives**

108 We aimed to explore the association between higher vancomycin MIC and clinical
109 outcome among patients with left-sided MSSA IE in the International Collaboration
110 Endocarditis (ICE) Cohort and whether HMV was identifiable by a genetic “signature” of
111 specific polymorphisms and virulence factors. The aims of this study were to validate the
112 findings of our previous study in the and to analyze whether specific genetic
113 characteristics were associated with the presence of *high* vancomycin MIC in a large,
114 well-characterized cohort of geographically diverse MSSA isolates causing IE.

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METHODS

Database

ICE-Pro prospective Cohort Study has been described previously [6]. Briefly, participating members from 64 sites in 28 countries reported patients prospectively on a standard case report form from 2000 through 2006. The case report form included 275 variables and was developed by ICE collaborators according to standard definitions [1]. All patients were included from sites that met performance criteria for participation. These criteria include the following: minimum enrolment of 12 patients per year in a centre with access to cardiac surgery; the presence of patient identification measures to ensure consecutive enrolment and to minimize ascertainment bias [1]; high quality data with query resolution, and the strains obtained from IE episodes were available in the ICE-Microbiology Repository [7], which included >1300 bloodstream isolates from prospectively identified patients with definite IE from 16 countries, obtained between June 2000 and September 2006. All participating sites had institutional review board or ethical committee approval or a waiver and informed consent (verbal or written) from all patients based on local standards as required by the Coordinating centre (Duke University Medical Center).

Study Sample

Patients and Settings

IE isolates were obtained from the Microbiological Repository of ICE-PCS [7]. Subjects in the ICE-Microbiology Repository cohort with available frozen bloodstream isolates with definite MSSA IE treated only with beta-lactams were eligible for inclusion in this study. Seventeen of these strains were included in the Spanish cohort [5]. Variables collected included demographics, characteristics of the episodes of IE, complications, treatment,

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3 156 and outcome. All patients who survived had, at least, one year of follow-up. Antibiotic
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5 157 therapy was decided by the treating physician at the individual study site.
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9 159 **Definitions**

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11 160 IE was defined according to the modified Duke Criteria [8] and was considered to be left-
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13 161 sided if no right-sided (tricuspid or pulmonary valve) vegetations were present on
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15 162 echocardiographic examination, surgery, or autopsy. A strain was considered to have
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17 163 HVM when it was ≥ 1.5 mg/L by E-test, and a low vancomycin MIC (LVM) when it was
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19 164 < 1.5 $\mu\text{g/mL}$. The rest of definitions have been previously provided in detail [9].
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25 166 **Geographic regions**

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27 167 Geographic regions included the following 25 participating sites (see Appendix: United
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29 168 States (4 sites), South America (2 sites from Brazil and Chile), Europe (12 sites from
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31 169 Croatia, France, Greece, Italy, Slovenia and Spain), Australia and New Zealand (6 sites)
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33 170 and Middle East (1 site from Lebanon).
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38 172 **Microbiological methods**

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40 173 ICE methodology for microbiological procedures has been defined elsewhere [6,10].
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42 174 Detailed microbiological methods can be found in the Supplemental Appendix. MSSA
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44 175 strains were stored at -80°C in a central laboratory (Duke University) and tested for
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46 176 nafcillin, cloxacillin, vancomycin and daptomycin MIC by E-test, following the
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48 177 manufacturer's recommendations (bioMérieux, Marcy l'Etoile, France).
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51 178 **Multiplex Polymerase Chain Reaction.** Genomic DNA was prepared as previously
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53 179 described [12]. Bacterial determinants including adhesins, toxins, *agr* group I–IV, and
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3 180 other genes were screened by multiplex polymerase chain reaction (PCR) as previously
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5 181 described [12]. All negative calls on the multiplex PCR were confirmed by uniplex PCR.
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10 183 **Spa Typing.** Spa typing was performed as previously described [12, 15]. PCR
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12 184 oligonucleotide primers for *spa* were described previously [15]. Samples were sequenced
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14 185 at the Duke University sequencing laboratory. For *spa* typing, eGenomics software
15
16 186 (<http://tools.egenomics.com/>) was used to scan the primary sequence to help identify the
17
18 187 orders and names of each repeat. The *spa* type number is representative of the repeat
19
20 188 organization. Clonal complexes (CC) for the isolates were identified via repeats pattern
21
22 189 recognition from existing *spa* type and CC database previously confirmed via multilocus
23
24 190 sequence typing (MLST). Isolates whose *spa* type did not map to a known CC underwent
25
26 191 MLST typing. For MLST, the sequence chromatograms for unique alleles were deposited
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28 192 in the MLST database (<http://www.mlst.net>). Alleles at the 7 loci (*arcC*, *aroE*, *glpF*, *gmk*,
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30 193 *pta*, *tpi*, and *yqiL*) were used to identify a unique sequence type (ST). MLST allele names
31
32 194 and STs were derived from <http://www.mlst.net>. CCs were assigned to groups of isolates
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34 195 sharing 6 of 7 alleles by using the eBURST algorithm (<http://eburst.mlst.net>) [16].
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39 40 41 197 **Statistical analysis**

42
43 198 Categorical variables were expressed as percentages and compared with Fisher's exact
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45 199 test. Continuous variables were expressed as means or medians (depending on the
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47 200 variable's homogeneity) and compared by non-parametric Wilcoxon's score/Mann
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49 201 Whitney's U tests. Survival analysis was performed by Kaplan-Meier analysis, and curves
50
51 202 were compared by the log-rank test. Given the limited sample size, logistic regression
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53 203 analysis was not performed. A two-sided p-value <0.05 was considered to be statistically
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55 204 significant. To determine the rate of type I errors in null hypothesis testing when
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205 conducting multiple comparisons among sociodemographic variables, the false discovery
206 rate (FDR) was calculated after adjustment at $P < 0.10$.

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RESULTS

Cases included

There were 1,072 cases of MSSA IE cases included in the ICE-PCS, of which 114 isolates were available in the ICE-Microbiology Repository substudy. Of these, 62 (28 LVM, 34 HVM) were cases of left-sided MSSA treated with anti-staphylococcal beta-lactam antibiotics with enough clinical data for the final analysis. The study flowchart is shown in **Figure 1**.

Vancomycin MIC distribution in the cohort

The distribution of vancomycin MIC among the 62 cases included in the study is displayed in **Figure 2** along with the respective rates of mortality for 0.75, 1, 1.5 and ≥ 2 $\mu\text{g/mL}$ vancomycin MIC determinations.

Demographics and clinical characteristics

Demographics and clinical characteristics of patients with LVM and HVM are shown in **Table 1**. There were no significant differences found in age, sex, place of acquisition of the infection, or geographical area between the two groups. Most episodes were native valve IE (77.4%) acquired in the community (64.5%). After adjustment, no variables were significant at FDR < 0.10 .

Outcomes

Complications, surgical rates and mortality according to vancomycin MIC groups are shown in **Table 1**. Differences between the two groups did not reach statistical significance in any of the variables. For LVM, in-hospital and one-year mortality were

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3 23 32% (9/28) and 43% (12/28), respectively, while in-hospital mortality was 27% (9/34) and
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5 239 one-year mortality 29% (10/34) for HVM.

240 Genotypic characteristics

241 Differences in genotypic characteristics between the two groups according to MIC
242 phenotypes are shown in the **Supplemental Appendix, Table 2**. No differences were
243 detected regarding adhesins, toxins or other putative virulence factors. Thirty-eight
244 percent of patients with HVM presented *agr-II* compared to 19% of patients with LVM.
245 However, this difference was not statistically significant ($P=0.157$). With regard to CC,
246 CC30 was the most frequent CC in the whole cohort. Differences in the prevalence of the
247 CCs between the HVM and LVM groups were not statistically significant.

249 Risk factors for mortality

250 The univariate analysis of risk factors for one-year mortality is shown in **Table 2**. HVM,
251 type of *agr* and CC were not significantly associated with mortality. The analysis for in-
252 hospital mortality using the same variables did not differ from that of one-year mortality
253 (data not shown). Among clinical markers, paravalvular complications [OR 4.0 (95%CI
254 1.1-14.6); $P=0.021$] and stroke [OR 4.72 (95%CI 1.2-19.1); $P=0.015$] were associated
255 with a poorer prognosis at one-year. With regard to virulence factors, see [OR 3.33 (0.9-
256 12.2); $P=0.044$] and *sei* [OR 2.94 (1.1-13.7); $P=0.044$] were identified as risk factors for
257 mortality whereas *chp* was found to be a protective factor [OR 0.07 (0-0.7); $P=0.006$].

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259 The Kaplan-Meier survival plot at one-year according to vancomycin MIC group is
260 displayed in the **Supplemental Appendix**.

Discussion

Vancomycin MIC was not associated with complications or mortality among patients with MSSA left-sided IE treated with beta-lactams. Thus, this study was unable to validate findings from the Cervera et al study [5]. Holmes et al. described significantly higher 30-day mortality in patients with MSSA bacteremia caused by strains with HVM, regardless of the treatment received (either cloxacillin or vancomycin) [7]. Another study found that HVM was significantly associated with a higher likelihood of developing a complicated bacteremia in a cohort of 99 patients with MSSA catheter-associated bacteremia [8]. In an 84-patient MSSA bacteremia cohort study, HVM was significantly related with *agr* dysfunction and *agr* type II polymorphism [9]. In the Cervera et al study we found MSSA endocarditis caused by strains with a HVM had a 3-fold higher in-hospital mortality rate and that major embolic events, especially stroke, were more frequent within this subgroup [11], from which two main hypotheses were raised: 1) HVM in MSSA isolates was associated with higher mortality in patients with left-sided IE due to an increased rate of major embolic events; and 2) a genomic signature identified MSSA isolates with HVM. However, in the present study systemic embolic events were higher in the LVM group and stroke was almost equally common in the two groups. Moreover, both in-hospital and one-year mortality were higher in the LVM group although these differences were not statistically significant probably because of the small sample size.

Main findings from studies investigating the relationship between VAN MIC and prognosis of MSSA and IE, as well as its relationship with *agr* dysfunction are shown in Table 3. Higher mortality among patients with LVM than in patients with HVM was found in the present study, as well as higher rates of systemic embolic events (36% vs. 23%) and persistent bacteremia (14% vs.12%), neither of them reaching statistical significance.

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3 288 Given that no significant differences were found in virulence factors between the two
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5 289 groups, we might speculate that in this dataset, due to the high proportion of patients with
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7 290 MSSA strains harbouring LVM and CC30, a high rate of detected and not detected
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9 291 hematogenous complications in the LVM may explain the higher mortality in this group.
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14 293 With regard to genotypic features, the analysis of *agr* subgroup did not reveal a
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16 294 significant association between a specific *agr* polymorphism and HVM or CC. We
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18 295 expected to find an association between a HVM and *agrII* polymorphism relying on
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20 296 previous studies performed in MSSA bacteremia [3,11]. Holmes et al. found an overall
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22 297 statistically significant association between a HVM and *agrII* in a cohort of 242 patients
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24 298 with *S. aureus* bacteremia (104 MRSA and only 38 MSSA) [17]. However, a subanalysis
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26 299 of the MSSA subset was not performed but would have probably been limited by small
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28 300 sample size. A more recent study that included 135 episodes of MSSA bacteremia of
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30 301 various origins did not find significant differences on *agr* subgroup and function according
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32 302 to the vancomycin MIC with a threshold of ≥ 1.5 $\mu\text{g/mL}$ [10]. In our study, *agrII* was two
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34 303 times more frequent in the HVM than in the LVM (38% vs. 19%), but did not reach
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36 304 statistical significance. In our cohort, CC5 was only slightly more common in patients with
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38 305 HVM and CC30 was two times more common in the LVM group. CC30 was the most
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40 306 common CC within the whole cohort as previously described in a cohort of 228 patients
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42 307 with MSSA IE and skin and STI [6]. As in our study, Viedma et al. did not find specific
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44 308 associations between CC subtypes and HVM although in their cohort there was a trend
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46 309 toward a higher proportion of CC5 in the HVM group compared to the LVM group (21.8%
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48 310 vs. 6.9%; $P=0.070$). Fowler and colleagues found a significantly higher proportion of CC5
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50 311 (and CC30) among patients with *S. aureus* bacteremia with hematogenous complications
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52 312 but not in the subgroup of MSSA patients [15]. In the study by Holmes et al [17], CC8
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3 313 was significantly associated with HVM, and in the case of CC5, the association
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5 314 approached statistical significance (6.5% in the LVM group and 14.2% in the HVM group;
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7 315 $P=0.055$). Thus, the hypothesis of a higher embolic potential among HVM strains could
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9 316 not be demonstrated. However, Further studies are needed before firm conclusions can
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11 317 be drawn regarding the association between CC and hematogenous complications in left-
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14 318 sided IE caused by MSSA strains.
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18 320 We did not identify a specific repertoire of virulence factors in the HVM group, as other
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20 321 previous studies did [6,7,10]. The virulence factors we found to be associated with worse
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22 322 prognosis have not been previously described as risk factors for mortality in the clinical
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24 323 field. In the Nienaber et al study, MSSA IE strains were shown to more likely contain 3
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26 324 adhesins (*clfB*, *cna*, *map/eap*) and 5 enterotoxins (*tst*, *sea*, *sed*, *see*, and *sei*) than STI
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28 325 strains with no significant geographical variations [12]. Holmes et al found a significantly
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30 326 higher proportion of *sea* and *clfA* among *S. aureus* strains with HVM causing bacteremia
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32 327 [17]. In a recently published Indian study, the most frequently identified toxin genes
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34 328 among patients with MSSA bacteremia were *sei* and *seg* [18]. In an epidemiologic study
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36 329 analyzing CC and diversity of exotoxin gene profiles performed in 81 strains of *S. aureus*
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38 330 isolated in a Spanish hospital (68% of which were MSSA), *agrII* was the most common
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40 331 gene and was linked to CC5, *sea* and *sec* [19]. In the study by Nienaber et al., no
41
42 332 significant *agrII* variations among geographical areas where found, with 68% of isolates
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44 333 being from Europe and Middle East, 23% from North America and 9% from Oceania [12].
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46 334 Although geographical regions are almost equally represented, changes in the genetic
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48 335 expression and phenotypic pattern in MSSA might be common over the time and
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50 336 geographical variations are also likely to occur as years pass. In the present cohort, the
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52 337 geographical regions are almost equally represented. However, since changes in the
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3 338 genetic expression and phenotypic pattern in MSSA might be common over the time and
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5 339 geographical variations are also likely to occur as years pass, we wonder if some of the
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7 340 different findings between the Cervera et al. study [5] and the present study could be due
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9 341 to different periods of time (1995-2011 in the Spanish cohort and 2000-2006 in the ICE)
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11 342 and geographic regions (local vs. intercontinental samples). Actually, the proportion of
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13 343 MSSA strains with HMV in Cervera et al. study significantly decreased along the period of
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15 344 the study [5]. Another factor that could also have influence in the percentage of MSSA
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17 345 strains identified as HVM is the effect of freezer storage. Ludwig et al. demonstrated a
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19 346 progressive decline of VAN MIC determination by Etest in MRSA bloodstream samples
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21 347 from the moment they were frozen [14]. Either due to locoregional or global variations of
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23 348 MSSA clones, further studies on the occurrence of variations of MSSA genotypic features
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25 349 are needed to correlate our results with more precise data.
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32 351 The superantigen genes *see* and *sei*, encoded by the enterotoxin gene cluster, conferred
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34 352 worse prognosis at one year, with 3-fold higher mortality. There are some *in vivo* data
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36 353 pointing to an important role of these virulence genes in the severity of invasive *S. aureus*
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38 354 infections [14]. However, the pathway in which these genes are involved, as other
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40 355 superantigens, is T-cell activation by binding to major histocompatibility complex class II
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42 356 molecules and to certain V β varieties on the T-cell receptor, leading to septic shock and
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44 357 not embolic events, including stroke. Chemotaxis inhibitory protein (*chp*), a protective
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46 358 factor in this cohort, is a major staphylococcal defense evasion mechanism against the
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48 359 human innate immune system [21]. Potentially, the presence of *chp* involves a loss of
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50 360 fitness and thus lowers virulence in patients with MSSA strains harboring this gene in this
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52 361 cohort. Yet, it is important to stress that outcomes of IE patients may not be easily
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54 362 attributable to simply virulence factors. The role played by the host is crucial and definite
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3 363 conclusions should be drawn from studies analyzing both sides of the interplay, namely
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5 364 the host and the pathogen. For example, outcomes of patients with IE due to relatively
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7 365 low-virulence microorganisms, such as coagulase-negative staphylococci, are associated
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9 366 with heart failure, the presence of prosthetic devices, comorbidities of the patient and the
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11 367 need of surgery [15-17] albeit coagulase-negative staphylococci causing IE have unique
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13 368 genetic signatures which are found across vast geographic distances [18], and HMV has
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15 369 also been identified as a marker of bad prognosis [19].
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21 371 This study has several limitations. First, the low sample size is a major shortcoming,
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23 372 severely limiting the statistical power of the study and leading to the potential for type II
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25 373 error in the analysis. Second, the number of isolates tested and available is not
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27 374 representative of the overall ICE dataset. However, we conducted a subanalysis
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29 375 comparing ICE left-sided MSSA frozen strains and those that were not frozen, and we did
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31 376 not detect significant differences regarding in-hospital and one-year mortality and surgery
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33 377 rates (data not shown). Third, *agrII* dysfunction was not measured, so correlations with
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35 378 *agr* subgroup, vancomycin MIC, and outcomes were not performed. Fourth, only cases
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37 379 occurring in the 2000-2006 period were included, which precluded observations
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39 380 regarding the temporal trends on vancomycin MIC, CC, or virulence factors according to
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41 381 each geographical region. Given that the data presented are older than 10 years, the
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43 382 clinical relevance of current MSSA
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45 383 circulating strains may have changed. Fifth, the potential variations of MSSA clones
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47 384 between geographical regions were not investigated. And finally, all patients were treated
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49 385 with anti-staphylococcal beta-lactam antibiotics (cloxacillin, nafcillin and first generation
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51 386 cephalosporins), but the specific type of beta-lactam used was not available. Despite
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53 387 these limitations, this is the first study on this subject including genetic studies of the
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3 388 bacterial isolates in order to find an association with the vancomycin phenotype and
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5 389 clinical outcomes.
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10 391 In conclusion, in this international cohort of left-sided MSSA IE treated with beta-lactams,
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12 392 vancomycin MIC ≥ 1.5 $\mu\text{g/mL}$ was not found to be an independent risk factor for
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14 393 complications of IE or for mortality. Stroke, paravalvular complications, and some *S.*
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16 394 *aureus* genes were associated with a worse outcome. Differences in clonality and
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18 395 virulence factors were not found between strains with LVM and HVM. Further studies in
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20 396 this field are warranted to expand upon these findings and elucidate if the contradictory
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22 397 results obtained in this field [15] are due to methodological limitations or rather to a
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25 398 difficult-to-interpret phenomenon.
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3 400 **Financial disclosures**

4 401 VGF reports the following potential conflicts of interest: Chair of the Scientific Advisory
5
6 402 Board for Merck V710; paid consultant for Pfizer, Novartis, Galderma, Novadigm, Durata,
7
8 403 Debiopharm, Genentech, Achaogen, Affinium, Medicines Co., Cerexa, Tetrphase, Trius,
9
10 404 MedImmune, Bayer, Theravance, Cubist, Basilea, Affinergy; Grants pending from
11
12 405 MedImmune, Actavis/Forest/Cerexa, Pfizer, Merck/Cubist, Advanced Liquid Logics,
13
14 406 Theravance, Novartis, Medical Surfaces, and Locus Biotechnology; royalties from
15
16 407 UpToDate, personal fees for development or presentation of educational presentations
17
18 408 (Green Cross, Cubist, Cerexa, Durata, Theravance), outside the submitted work; and
19
20 409 patent pending related to sepsis diagnostics. JMM has received consulting honoraria
21
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24 411 Sciences, and ViiV. All other authors: no conflicts.
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574 **FIGURE LEGENDS**

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576 **Figure 1.** Flowchart of the patients included in the study. Abbreviation: ICE, International
577 Collaboration on Endocarditis; IE, infective endocarditis; MIC, Minimum Inhibitory
578 Concentration; MSSA, methicillin-susceptible *Staphylococcus aureus*; VAN, vancomycin.

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580 **Figure 2.** Distribution of the vancomycin MIC within the cohort of 62 left-sided MSSA
581 Infective endocarditis and overall one-year mortality rates according to vancomycin MIC
582 determination.

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584 **Figure 3.** Kaplan–Meier survival graph of mortality at one-year days of follow-up according
585 to vancomycin minimum inhibitory concentration. Abbreviation: MIC, minimum inhibitory
586 concentration.

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Table 1. Demographics, Clinical Characteristics and Outcomes of 62 Episodes of Left-sided Methicillin-Susceptible *Staphylococcus aureus* Infective Endocarditis Treated with Beta-lactams According to “High” or “Low” Vancomycin Minimum Inhibitory Concentration (MIC).

	Vancomycin MIC <1.5 µg/mL n=28	Vancomycin MIC ≥1.5 µg/mL n=34	P
Mean age (SD)	61.1 (18.5)	60.1 (14.3)	0.396
Male gender (%)	19 (68%)	27 (79%)	0.386
Type of endocarditis:			0.548
▪ Native valve	23 (82%)	25 (74%)	
▪ Prosthetic valve	5 (19%)	9 (27%)	
Origin of acquisition:			0.916
▪ Community-acquired	17 (61%)	23 (68%)	
▪ Nosocomial	8 (29%)	9 (26%)	
▪ Healthcare-associated	2 (7%)	2 (6%)	
▪ Unknown	1 (3%)	0	
Geographical area:			0.333
▪ North America	4 (14%)	10 (29%)	
▪ Europe/Mid East	20 (71%)	22 (65%)	
▪ South America	1 (4%)	0	
▪ Australia/New Zealand	3 (11%)	2 (6%)	
Complications:			
▪ Heart failure	13 (46%)	10 (29%)	0.195
▪ Systemic emboli	10 (36%)	8 (23%)	0.400
▪ Stroke	7 (25%)	9 (27%)	1.000
▪ Paravalvular complications	8 (29%)	11 (32%)	0.788
▪ New conduction abnormality	0 (-)	3 (9%)	0.245
▪ Persistent bacteremia	4 (14%)	4 (12%)	1.000
Surgical treatment	9 (32%)	14 (41%)	0.599
Relapses [‡]	0	0	1.000

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Mortality			
▪ In-hospital mortality	9 (32%)	9 (27%)	0.780
▪ One-year mortality	12 (43%)	10 (29%)	0.298

* Defined as a new episode of endocarditis due to the same microorganism that caused the first IE within the following 12 months.

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Table 2. Genotypic Characteristics and Distribution of Clonal Complexes of 62 Episodes of Left-sided Methicillin-Susceptible *Staphylococcus aureus* Infective Endocarditis Treated with Beta-lactams According to High or Low Vancomycin Minimum Inhibitory Concentration (MIC).

	Vancomycin MIC <1.5 µg/mL n=28	Vancomycin MIC ≥1.5 µg/mL n=34	P
Adhesins			
- <i>fnbA</i>	28 (100%)	34 (100%)	1.000
- <i>fnbB</i>	5 (19%)	7 (21%)	1.000
- <i>clfA</i>	28 (100%)	34 (100%)	1.000
- <i>clfB</i>	28 (100%)	34 (100%)	1.000
- <i>cna</i>	25 (90%)	29 (85%)	0.720
- <i>spa</i>	27 (96%)	33 (97%)	1.000
- <i>scrC</i>	12 (43%)	16 (47%)	0.801
- <i>sdrD</i>	18 (64%)	19 (56%)	0.606
- <i>sdrE</i>	12 (43%)	16 (47%)	0.801
- <i>bbp</i>	25 (89%)	28 (82%)	0.495
- <i>ebpS</i>	28 (100%)	34 (100%)	1.000
- <i>map/eap</i>	17 (61%)	25 (74%)	0.413
Toxins			
- <i>eta</i>	4 (14%)	10 (29%)	0.225
- <i>etb</i>	1 (4%)	1 (3%)	1.000
- <i>tst</i>	27 (96%)	31 (91%)	0.620
- <i>sea</i>	21 (75%)	19 (56%)	0.182
- <i>seb</i>	0	2 (6%)	0.497
- <i>sec</i>	6 (21%)	10 (29%)	0.566
- <i>sed</i>	5 (18%)	9 (27%)	0.546
- <i>see</i>	9 (32%)	9 (27%)	0.780
- <i>seg</i>	22 (79%)	24 (71%)	0.566
- <i>seh</i>	1 (4%)	4 (12%)	0.366

- <i>sej</i>	24 (86%)	31 (91%)	0.691
- <i>sej</i>	1 (4%)	2 (6%)	1.000
- <i>pvi</i>	6 (21%)	3 (9%)	0.277
- <i>hlg</i>	28 (100%)	34 (100%)	1.000
Other putative virulence genes			
- <i>efb</i>	28 (100%)	34 (100%)	1.000
- <i>icaA</i>	24 (86%)	31 (91%)	0.691
- <i>chp</i>	25 (89%)	30 (88%)	1.000
- V8	9 (32%)	19 (56%)	0.077
Agr subgroup			
- I	10 (37%)	12 (35%)	1.000
- II	5 (19%)	13 (38%)	0.157
- III	12 (44%)	8 (24%)	0.105
- IV	0	1 (3%)	1.000
Clonal Complex			
- CC1	2 (7%)	1 (3%)	0.579
- CC5	4 (15%)	6 (18%)	1.000
- CC8	2 (7%)	2 (6%)	1.000
- CC15	1 (4%)	4 (12%)	0.371
- CC30	8 (30%)	5 (15%)	0.212
- CC45	5 (19%)	6 (18%)	1.000
- Other	5 (19%)	10 (29%)	0.382

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Table 2. **Bi-variable** Analysis of Risk Factors for One-year Mortality

Variable	Alive n=39	Dead n=22	OR (95%CI)	<i>P</i>
Demographics				
Age >65years	16 (40%)	11 (50%)	1.5 (0.5-4.9)	0.593
Male sex	31 (78%)	15 (68%)	0.62 (0.2-2.4)	0.546
Prosthetic valve IE	11 (29%)	3 (14%)	0.41 (0.1-1.9)	0.338
Diabetes	8 (20%)	4 (18%)	0.89 (0.2-3.9)	1.000
Acquisition in the community	26 (65%)	14 (64%)	1.76 (0.0-141.9)	0.889
North America	8 (20%)	6 (27%)	1.77 (0.1-143.1)	0.444
Clinical features				
Paravalvular complications	8 (20%)	11 (50%)	4.0 (1.1-14.6)	0.021
Stroke	6 (15%)	10 (46%)	4.72 (1.2-19.1)	0.015
Heart failure	10 (46%)	10 (46%)	1.73 (0.5-5.7)	0.411
Persistent bacteremia	5 (13%)	3 (14%)	1.11 (0.2-6.4)	1.000
Surgery	14 (35%)	9 (41%)	1.29 (0.4-4.2)	0.785
Microbiological features				
Vancomycin MIC \geq 1.5 mg/L	24 (60%)	10 (45%)	0.56 (0.2-1.8)	0.298
CC30	7 (18%)	6 (27%)	1.77 (0.4-7.3)	0.516
CC8	1 (3%)	3 (14%)	6.16 (0.4-331.3)	0.546
CC15	6 (15%)	0	0.0 (0-1.1)	0.081
AGR subgroup I	15 (38%)	7 (32%)	0.78 (0.2-2.6)	0.784
AGR subgroup II	13 (33%)	6 (27%)	0.78 (0.2-2.7)	0.777
AGR subgroup III	11 (28%)	9 (41%)	1.83 (0.5-6.2)	0.394
<i>See</i>	8 (20%)	10 (46%)	3.33 (0.9-12.2)	0.044
<i>Sei</i>	33 (83%)	22 (100%)	2.94 (1.1-13.7)	0.044
<i>Chp</i>	39 (98)	16 (73%)	0.07 (0.0-0.7)	0.006
<i>Eta</i>	8 (20%)	6 (27%)	1.5 (0.4-5.9)	0.539
<i>Pvl</i>	4 (10%)	5 (23%)	2.65 (0.5-14.9)	0.259

The analysis for in-hospital mortality using the same variables did not differ from that of one-year mortality (data not shown).

Table 3. Summary of Main Findings from Studies Assessing the Relationship between Vancomycin MIC and Prognosis in MSSA Bacteremia and Left-sided IE.

First author, year	Design	SAB/IE (n)	Overall mortality Low VAN MIC < 1.5 µg/mL	Overall mortality High VAN MIC ≥ 1.5 µg/mL	Genetic factors	Main outcomes analysis
Kalil, 2014 [15]	Systematic review and metaanalysis	SAB (8291, both MRSA and MSSA)	25.8% (1430/5551)	26.8% (734/2740)	NA	RD 1.6% [95%CI, -2.3% to 5.6%]; P = 0.43 (for absolute risk of mortality, combining 30-day mortality and in-hospital mortality)
Holmes, 2011 [1]	Prospective multicenter cohort study	SAB (532; 266 of which MSSA treated with betalactams only)	12.2% (24/193)	26.8% (18/68)	NA	P= 0.011 (for 30-day mortality)
Holmes, 2014 [11]	Analysis of a subset of strains from [1]	SAB (252 MSSA isolates)	NA	NA	Associated with HVM: CC8, <i>agr</i> dysfunction, <i>agr</i> genotype II, <i>blaZ</i> , <i>sea</i> , <i>clfA</i> , <i>spIA</i> and ACME locus. Associated to LVM: CC22, CC88 and CC188	Associated with HVM: CC8 P < 0.001), <i>agr</i> dysfunction (P= 0.014), <i>agr</i> genotype II (P=0.043), <i>blaZ</i> (P=0.002), <i>sea</i> (P < 0.001), <i>clfA</i> (P < 0.001), <i>spIA</i> (P < 0.001) and ACME locus (P=0.02). Associated with LVM: CC22 (P < 0.001), CC88 (P < 0.001) and CC188 (P=0.002)
Aguado, 2011 [2]	Retrospective, single-center Cohort	Catheter-related SAB (99, all MSSA)	10.5% (8/76)	26.1% (6/23)	NA	P=0.13 (for 30-day mortality) OR=22.9, [95%CI 6.7–78.1] for complicated SAB.

López-Cortes, 2015 [4]	Prospective, single-center cohort	SAB (135, all MSSA)	23.6% (25/106)	10.3% (3/29)	There were no differences in <i>agr</i> distribution or absence of δ -haemolysin between isolates with HVM and those with LVM. HVM was not more frequent in specific clones.	RR=0.44 [95%CI, 0.14-1.35] for 14-day mortality
Viedma, 2014 [3]	Retrospective, single-center cohort	SAB (84, all MSSA)	24.1%, (7/29) <i>agr II</i> polymorphism: 17.2%; average levels of RNAIII gene expression: Δ Ct 1.5 \pm 2.11	45.5% (25/55) <i>agr II</i> polymorphism: 41.8%; average levels of RNAIII gene expression: Δ Ct 4.05 \pm 3.29	HVM: <i>agr II</i> polymorphism: 17.2%; average levels of RNAIII gene expression: Δ Ct 1.5 \pm 2.11 LVM: <i>agr II</i> polymorphism: 41.8%; average levels of RNAIII gene expression: Δ Ct 4.05 \pm 3.29	In-hospital mortality: P=0.057 <i>agr</i> dysfunction: P=0.023. RNAIII expression: P<.01
Cervera, 2014 [5]	Prospective, single-center cohort	MSSA IE (93)	31% (16/53)	53% (21/40)	NA	In-hospital mortality: P=0.035; Patients with HVM presented significantly more severe embolic events
Current study	Prospective, multicenter cohort	MSSA IE (62)	32% (9/28)	27% (9/34)	HVM: <i>agrII</i> polymorphism: 19% LVM: <i>agrII</i> polymorphism: 38%	In-hospital mortality: P=0.780. <i>agrII</i> polymorphism: P=0.157

CI: Confidence interval; IE: Infective endocarditis; MRSA: methicillin-resistant *Staphylococcus aureus*; NA: not addressed; OR: Odds ratio; RD: relative difference; RR: relative risk; SAB: *S. aureus* bacteremia.

Supplement Appendix

1. Microbiological Methods

E-test for Vancomycin MIC

MSSA strains were stored at -80° C in a central laboratory (Duke University) and tested for vancomycin MIC by E-test after the freezing of the samples, following the manufacturer's recommendations (bioMérieux, Marcy l'Etoile, France). E-test were done by duplicate and interpreted by consensus with a second researcher.

Multiplex Polymerase Chain Reaction. Genomic DNA was prepared as previously described [1]. Bacterial determinants including adhesins, toxins, *agr* group I–IV, and other genes were screened by multiplex polymerase chain reaction (PCR) as described before [1]. All negative calls on the multiplex PCR were confirmed by uniplex PCR.

Spa Typing. Spa typing was performed as previously described [1, 2]. PCR oligonucleotide primers for *spa* were described previously [2]. Samples were sequenced at the Duke University sequencing laboratory. For *spa* typing, eGenomics software (<http://tools.egenomics.com/>) was used to scan the primary sequence to help identify the orders and names of each repeat. The *spa* type number is representative of the repeat organization. Clonal complexes (CC) for the isolates were identified via repeats pattern recognition from 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 typing. For MLST, the sequence chromatograms for unique alleles were deposited in the MLST database (<http://www.mlst.net>). Alleles at the 7 loci

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4 26 (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) were used to identify a unique
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6 27 sequence type (ST). MLST allele names and STs were derived from
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8 28 <http://www.mlst.net>. CCs were assigned to groups of isolates sharing 6 of 7
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10 29 alleles by using the eBURST algorithm (<http://eburst.mlst.net>) [3].
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44 2. Genotypic characteristics of MSSA strains according to VAN MIC.

45 45 Differences in genotypic characteristics between the two groups according to
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47 46 MIC phenotypes are displayed in the **Table 1**. No differences were detected
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49 47 regarding adhesins, toxins or other putative virulence factors. Thirty-eight
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51 48 percent of patients with HVM presented *agr*-II compared to 19% of patients with
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53 49 LVM. However, this difference was not statistically significant ($P=0.157$). With
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55 50 regard to CC, CC30 was the most frequent CC in the whole cohort. Differences
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in the prevalence of the CCs between the HVM and LVM groups were not statistically significant.

Table 1. Genotypic Characteristics and Distribution of Clonal Complexes of 62 Episodes of Left-sided Methicillin-susceptible *Staphylococcus aureus* Infective Endocarditis Treated with Beta-lactams According to High or Low Vancomycin Minimum Inhibitory Concentration (MIC).

	Vancomycin MIC < 1.5 µg/mL n=28	Vancomycin MIC ≥ 1.5 µg/mL n=34	<i>P</i>
Adhesins			
- <i>fnbA</i>	28 (100%)	34 (100%)	1.000
- <i>fnbB</i>	5 (19%)	7 (21%)	1.000
- <i>clfA</i>	28 (100%)	34 (100%)	1.000
- <i>clfB</i>	28 (100%)	34 (100%)	1.000
- <i>cna</i>	25 (90%)	29 (85%)	0.720
- <i>spa</i>	27 (96%)	33 (97%)	1.000
- <i>scrC</i>	12 (43%)	16 (47%)	0.801
- <i>sdrD</i>	18 (64%)	19 (56%)	0.606
- <i>sdrE</i>	12 (43%)	16 (47%)	0.801
- <i>bbp</i>	25 (89%)	28 (82%)	0.495
- <i>ebpS</i>	28 (100%)	34 (100%)	1.000
- <i>map/eap</i>	17 (61%)	25 (74%)	0.413
Toxins			
- <i>eta</i>	4 (14%)	10 (29%)	0.225
- <i>etb</i>	1 (4%)	1 (3%)	1.000
- <i>tst</i>	27 (96%)	31 (91%)	0.620
- <i>sea</i>	21 (75%)	19 (56%)	0.182
- <i>seb</i>	0	2 (6%)	0.497
- <i>sec</i>	6 (21%)	10 (29%)	0.566
- <i>sed</i>	5 (18%)	9 (27%)	0.546

- see	9 (32%)	9 (27%)	0.780
- seg	22 (79%)	24 (71%)	0.566
- seh	1 (4%)	4 (12%)	0.366
- sei	24 (86%)	31 (91%)	0.691
- sej	1 (4%)	2 (6%)	1.000
- pvl	6 (21%)	3 (9%)	0.277
- hlg	28 (100%)	34 (100%)	1.000
Other putative virulence genes			
- <i>efb</i>	28 (100%)	34 (100%)	1.000
- <i>icaA</i>	24 (86%)	31 (91%)	0.691
- <i>chp</i>	25 (89%)	30 (88%)	1.000
- V8	9 (32%)	19 (56%)	0.077
Agr subgroup			
- I	10 (37%)	12 (35%)	1.000
- II	5 (19%)	13 (38%)	0.157
- III	12 (44%)	8 (24%)	0.105
- IV	0	1 (3%)	1.000
Clonal Complex			
- CC1	2 (7%)	1 (3%)	0.579
- CC5	4 (15%)	6 (18%)	1.000
- CC8	2 (7%)	2 (6%)	1.000
- CC15	1 (4%)	4 (12%)	0.371
- CC30	8 (30%)	5 (15%)	0.212
- CC45	5 (19%)	6 (18%)	1.000
- Other*	5 (19%)	10 (29%)	0.382

* Other CC analysed comprised CC9, CC22, CC25, CC34, CC51, CC59, CC72, and 125.

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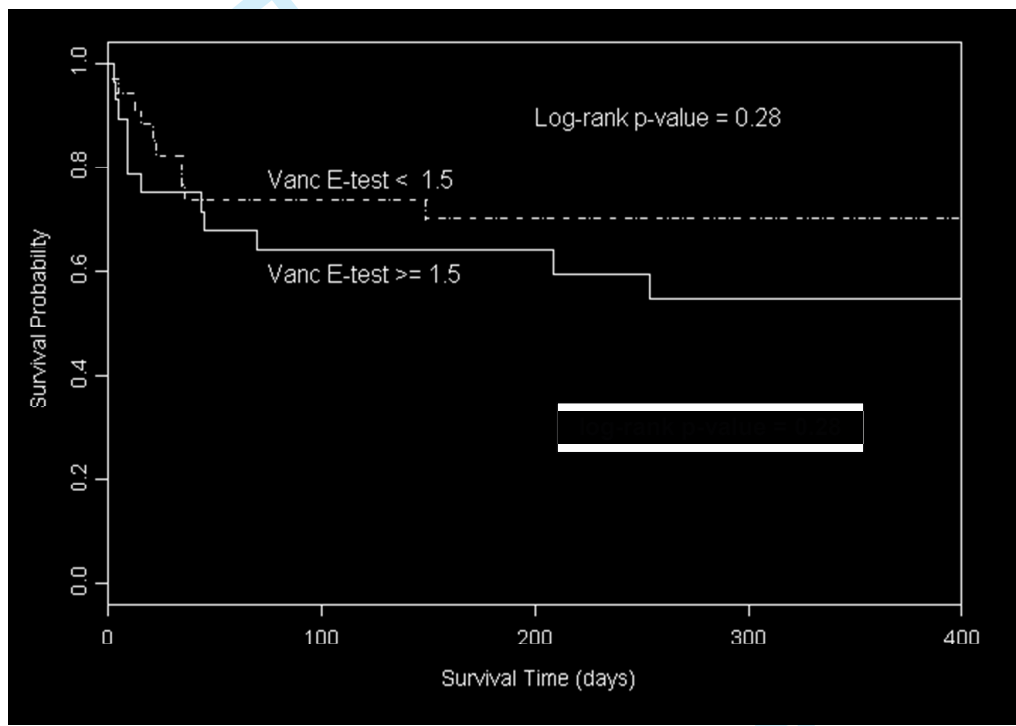
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Figure 1. Kaplan–Meier survival graph of mortality at one-year days of follow-up according to vancomycin minimum inhibitory concentration. Abbreviation: MIC, minimum inhibitory concentration.



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Title: Influence of Vancomycin Minimum Inhibitory Concentration on the Outcome of Methicillin-Susceptible *Staphylococcus aureus* Left-Sided Infective Endocarditis Treated with Anti-staphylococcal Beta-Lactam Antibiotics; a Prospective Cohort Study by the International Collaboration on Endocarditis.

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10 33 **Keywords:** *Staphylococcus aureus*, vancomycin MIC, endocarditis, phenotype,
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12 34 genotype.
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14 35 **Keypoints:** In this international cohort of patients with left-sided MSSA endocarditis
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16 36 treated with anti-staphylococcal beta-lactam antibiotics, vancomycin MIC phenotype was
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18 37 not associated with patient demographics, clinical outcome, or virulence gene repertoire.
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20 38 **Running title:** “Vancomycin MIC and MSSA endocarditis”.

21 39 **Word count:** 1,198

22 40 **Abstract word count:** 249

23 41 **References:** 15

24 42 **Tables:** 3

25 43 **Figures:** 2

26 44 **Supplementary Appendix:** 1 table, 1 figure and microbiological methods.

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43 53 #INT15/00168 during 2016.
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54 These data were partially presented at the 55th ICAAC, September 18-21, 2015. San
55 Diego, CA (abstract number 2423).

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For Peer Review

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3 **Abstract**
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58 **Objectives:** A recent study showed a poorer prognosis in left-sided MSSA endocarditis
59 treated with cloxacillin when the vancomycin MIC was ≥ 1.5 mg/L. We aimed to validate
60 these results using the International Collaboration on Endocarditis cohort and to analyze
61 whether specific genetic characteristics were associated with a high vancomycin MIC
62 (≥ 1.5 mg/L) phenotype.

63 **Methods:** All patients with left-sided MSSA IE treated with anti-staphylococcal beta-
64 lactam antibiotics between 2000 and 2006 with available isolates were included.
65 Vancomycin MIC (VAN MIC) was determined by E-test as either *high* (≥ 1.5 mg/L) or *low*
66 (< 1.5 mg/L). Isolates underwent *spa* typing to infer clonal complexes (CC) and multiplex
67 polymerase chain reaction for identifying virulence genes. Univariate analysis was
68 performed to evaluate the association between in-hospital and one-year mortality and
69 vancomycin MIC phenotype.

70 **Results:** 62 cases met the inclusion criteria. VAN MIC was *low* in 28 cases (45%) and
71 *high* in 34 cases (55%). No significant differences in patient demographic data or
72 characteristics of infection were observed between patients with IE due to *high* and *low*
73 VAN MIC isolates. Isolates with *high* and *low* VAN MIC had similar distributions of
74 virulence genes and clonal lineages. In-hospital and one-year mortality did not differ
75 significantly between the two groups (32% (9/28) vs. 27% (9/34), $P=0.780$; and 43%
76 (12/28) vs. 29% (10/34), $P=0.298$, for *low* and *high* VAN MIC, respectively).

77 **Conclusion:** In this international cohort of patients with left-sided MSSA endocarditis
78 treated with anti-staphylococcal beta-lactams, vancomycin MIC phenotype was not
79 associated with patient demographics, clinical outcome, or virulence gene repertoire.
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BACKGROUND

The impact of a *high* vancomycin MIC phenotype (HVM; ≥ 1.5 mg/L) in MSSA bacteremia and IE is poorly known. To date, several studies reported higher rates of complications and mortality in patients with MSSA bacteremia caused by strains with HVM [1,2], as well as a correlation with *agr* dysfunction and *agr* type II polymorphism [3]. Nonetheless, a more recent study did not find significant differences on *agr* subgroup and function according to the vancomycin MIC [4]. The association between HVM and significantly higher mortality was also demonstrated in a Spanish a cohort of 93 patients with MSSA IE treated with cloxacillin [5].

Objectives

We aimed to explore the association between higher vancomycin MIC and clinical outcome among patients with left-sided MSSA IE in the International Collaboration Endocarditis (ICE) Cohort and whether HVM was identifiable by a genetic “signature” of specific polymorphisms and virulence factors.

107 **METHODS**

108 **Database**

109 ICE-Pro prospective Cohort Study has been described previously [6]. All patients were
110 included from sites that met performance criteria for participation and the strains obtained
111 from IE episodes were available in the ICE-Microbiology Repository [7]. All participating
112 sites had institutional review board or ethical committee approval or a waiver and
113 informed consent from all patients based on local standards as required by the
114 Coordinating centre (Duke University Medical Center).

116 **Study Sample**

117 **Patients and Settings**

118 IE isolates were obtained from the Microbiological Repository of ICE-PCS [7]. Subjects in
119 the ICE-Microbiology Repository cohort with available frozen bloodstream isolates with
120 definite MSSA IE treated with beta-lactams were eligible for inclusion in this study.
121 Seventeen of these strains were included in the Spanish cohort [5]. All patients who
122 survived had, at least, one year of follow-up.

124 **Definitions**

125 IE was defined according to the modified Duke Criteria [8] and was considered to be left-
126 sided if no right-sided (tricuspid or pulmonary valve) vegetations were present on
127 echocardiographic examination, surgery, or autopsy. The rest of definitions have been
128 previously provided in detail [9].

130 **Geographic regions**

131 Twenty-five sites from all Geographic regions participated in the study (see **Appendix**)

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Microbiological methods

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ICE methodology for microbiological procedures has been defined elsewhere [6,10].

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Detailed microbiological methods can be found in the **Supplemental Appendix**.

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Statistical analysis

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Categorical variables were expressed as percentage and compared with Fisher's exact

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test. Continuous variables were expressed as means or medians and compared by non-

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parametric tests. Survival analysis was performed by Kaplan-Meier analysis, and curves

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were compared by the log-rank test. A two-sided p-value <0.05 was considered to be

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statistically significant.

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150 RESULTS

151 Cases included

152 The study flowchart is shown in **Figure 1**.

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154 Vancomycin MIC distribution in the cohort

155 The distribution of vancomycin MIC among the 62 cases included in the study is
156 displayed in **Figure 2** along with the respective rates of mortality.

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158 Demographics and clinical characteristics

159 Demographics and clinical characteristics of patients with LVM and HVM are shown in

160 **Table 1**.

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162 Outcomes

163 Complications, surgical rates and mortality according to vancomycin MIC groups are
164 shown in **Table 1**. Differences between the two groups did not reach statistical
165 significance in any of the variables. For LVM, in-hospital and one-year mortality were
166 32% (9/28) and 43% (12/28), respectively, while in-hospital mortality was 27% (9/34) and
167 one-year mortality 29% (10/34) for HVM.

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169 Genotypic characteristics

170 Differences in genotypic characteristics between the two groups according to MIC
171 phenotypes are shown in the **Supplemental Appendix**. No differences were detected
172 regarding adhesins, toxins or other putative virulence factors.

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174 Risk factors for mortality

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3 175 The univariate analysis of risk factors for one-year mortality is shown in **Table 2**. HVM,
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5 176 type of *agr* and CC were not significantly associated with mortality. The analysis for in-
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7 177 hospital mortality using the same variables did not differ from that of one-year mortality
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9 178 (data not shown).
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14 180 The Kaplan-Meier survival plot at one-year according to vancomycin MIC group is
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16 181 displayed in the **Supplemental Appendix**.
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3 200 **Discussion**
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5 201 Vancomycin MIC was not associated with complications or mortality among patients with
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7 202 MSSA left-sided IE treated with beta-lactams. Thus, this study was unable to validate
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9 203 findings from the Cervera et al study [5], from which two main hypotheses were raised: 1)
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11 204 HVM in MSSA isolates was associated with higher mortality in patients with left-sided IE
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13 205 due to an increased rate of major embolic events; and 2) a genomic signature identified
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15 206 MSSA isolates with HVM.
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20 208 Main findings from studies investigating the relationship between VAN MIC and prognosis
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22 209 of MSSA and IE, as well as its relationship with *agr* dysfunction are shown in **Table 3**.
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27 211 Higher mortality among patients with LVM than in patients with HVM was found in the
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29 212 present study, as well as higher rates of systemic embolic events (36% vs. 23%) and
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31 213 persistent bacteremia (14% vs.12%), neither of them reaching statistical significance.
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33 214 Given that no significant differences were found in virulence factors between the two
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35 215 groups, we might speculate that in this dataset, due to the high proportion of patients with
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37 216 MSSA strains harbouring LVM and CC30, a high rate of detected and not detected
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39 217 hematogenous complications in the LVM may explain the higher mortality in this group.
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45 219 With regard to genotypic features, the analysis of *agr* subgroup did not reveal a
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47 220 significant association between a specific *agr* polymorphism and HVM or CC. We
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49 221 expected to find an association between a HVM and *agrII* polymorphism relying on
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51 222 previous studies performed in MSSA bacteremia [3,11]. In our study, *agrII* was two times
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53 223 more frequent in the HVM than in the LVM (38% vs. 19%), but did not reach statistical
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55 224 significance.
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3 225 We did not identify a specific repertoire of virulence factors in the HVM group, as other
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5 226 previous studies did [6,7,10]. Although geographical regions are almost equally
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7 227 represented, changes in the genetic expression and phenotypic pattern in MSSA might
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9 228 be common over the time and geographical variations are also likely to occur as years
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11 229 pass. Another factor that could also have influence in the percentage of MSSA strains
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14 230 identified as HVM is the effect of freezer storage. Ludwig et al demonstrated a
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16 231 progressive decline of VAN MIC determination by Etest in MRSA bloodstream samples
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18 232 from the moment they were frozen [14].
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23 234 This study has several limitations. First, the low sample size is a major shortcoming,
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25 235 severely limiting the statistical power of the study and leading to the potential for type II
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27 236 error in the analysis. Second, the number of isolates tested and available is not
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29 237 representative of the overall ICE dataset. However, we conducted a subanalysis
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31 238 comparing ICE left-sided MSSA frozen strains and those that were not frozen, and we did
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33 239 not detect significant differences regarding in-hospital and one-year mortality and surgery
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35 240 rates (data not shown). Third, *agrII* dysfunction was not measured, so correlations with
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37 241 *agr* subgroup, vancomycin MIC, and outcomes were not performed. Fourth, only cases
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39 242 occurring in the 2000-2006 period were included, which precluded observations
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41 243 regarding the temporal trends Fifth, the potential variations of MSSA clones between
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43 244 geographical regions were not investigated. And finally, the specific type of beta-lactam
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45 245 used was not available.
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51 247 In conclusion, in this international cohort of left-sided MSSA IE treated with beta-lactams,
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53 248 vancomycin MIC ≥ 1.5 $\mu\text{g/mL}$ was not found to be an independent risk factor for
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55 249 complications of IE or for mortality. Stroke, paravalvular complications, and some *S.*
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3 250 *aureus* genes were associated with a worse outcome. Differences in clonality and
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5 251 virulence factors were not found between strains with LVM and HVM. Further studies in
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7 252 this field are warranted to expand upon these findings and elucidate if the contradictory
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9 253 results obtained in this field [15] are due to methodological limitations or rather to a
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12 254 difficult-to-interpret phenomenon.
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256 **Financial disclosures**

257 VGF reports the following potential conflicts of interest: Chair of the Scientific Advisory
258 Board for Merck V710; paid consultant for Pfizer, Novartis, Galderma, Novadigm, Durata,
259 Debiopharm, Genentech, Achaogen, Affinium, Medicines Co., Cerexa, Tetrphase, Trius,
260 MedImmune, Bayer, Theravance, Cubist, Basilea, Affinergy; Grants pending from
261 MedImmune, Actavis/Forest/Cerexa, Pfizer, Merck/Cubist, Advanced Liquid Logics,
262 Theravance, Novartis, Medical Surfaces, and Locus Biotechnology; royalties from
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267 Sciences, and ViiV. All other authors: no conflicts.

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3 395 **FIGURE LEGENDS**

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6 397 **Figure 1.** Flowchart of the patients included in the study. Abbreviation: ICE, International
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8 398 Collaboration on Endocarditis; IE, infective endocarditis; MIC, Minimum Inhibitory
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10 399 Concentration; MSSA, methicillin-susceptible *Staphylococcus aureus*; VAN, vancomycin.

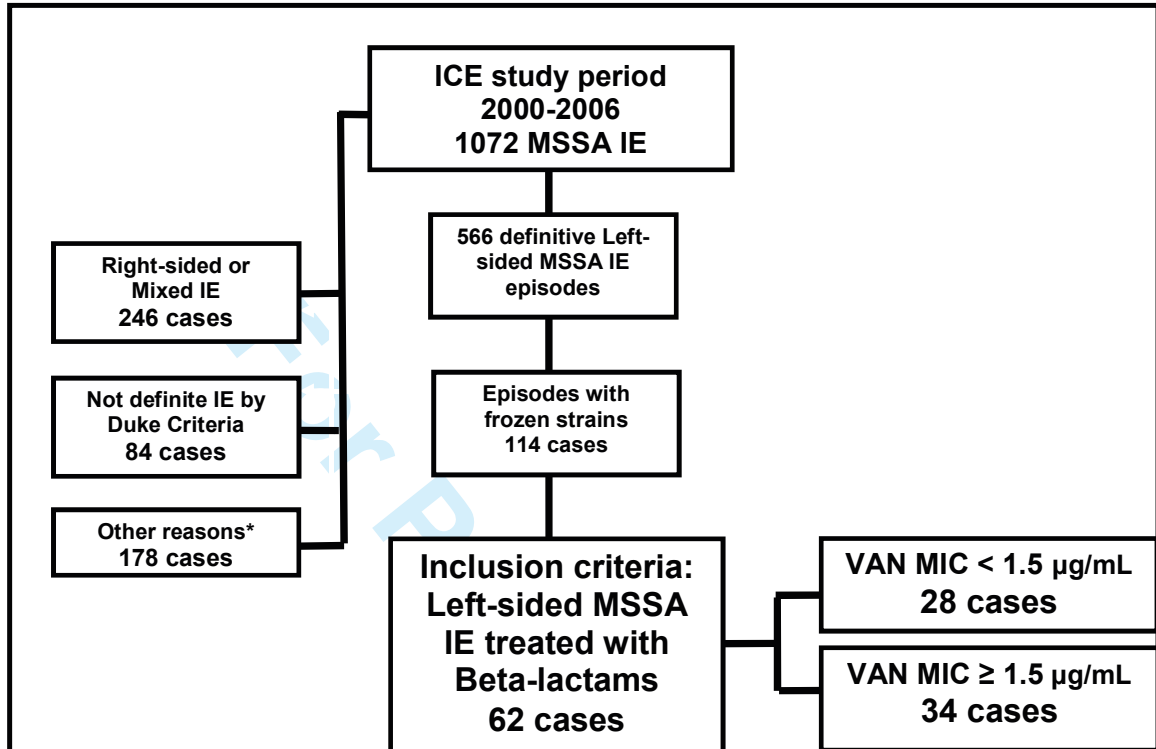
11 400
12
13 401 **Figure 2.** Distribution of the vancomycin MIC within the cohort of 62 left-sided MSSA
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15 402 Infective endocarditis and overall one-year mortality rates according to vancomycin MIC
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17 403 determination.

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For Peer Review

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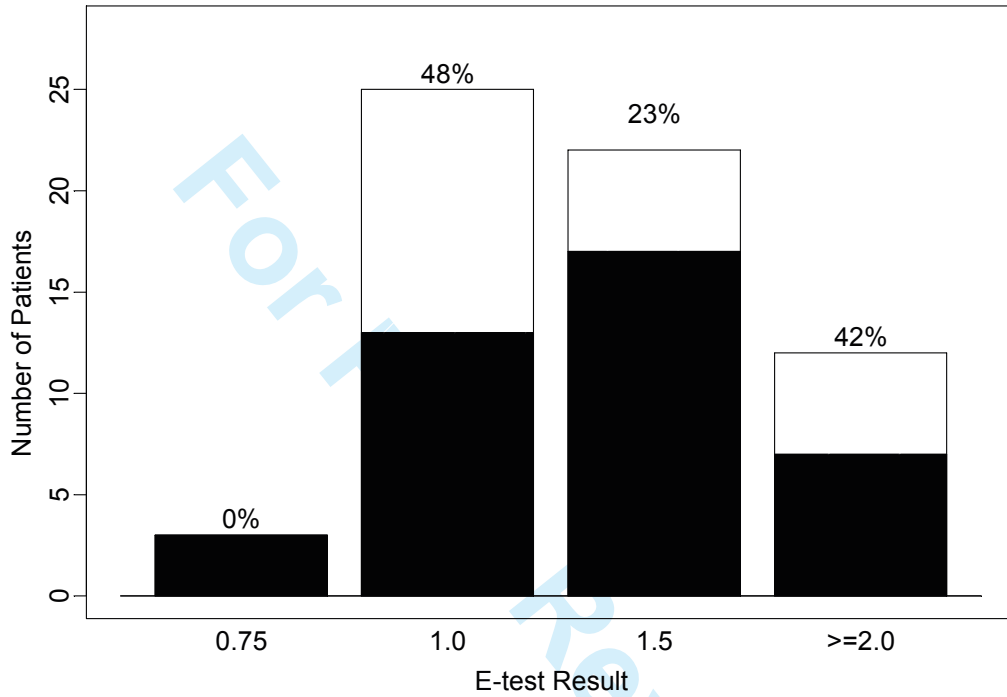
Figure 1.



*Other causes of exclusion were: lack of vegetations in the echocardiographic, surgical or post-mortem examination (123 patients), age below 18 years (7 patients) and relevant data missing in the CRD (48 patients).

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Figure 2.



VAN Group	0.75	1.0	1.5	≥ 2.0
N	3	25	22	12
Deaths	0	12	5	5

Percentages and white areas of every column indicate rates of one-year mortality per VAN MIC group. Black areas represent the percentage of patients alive at one-year.

Table 1. Demographics, Clinical Characteristics and Outcomes of 62 Episodes of Left-sided Methicillin-susceptible *Staphylococcus aureus* Infective Endocarditis Treated with Beta-lactams According to “High” or “Low” Vancomycin Minimum Inhibitory Concentration (MIC).

	Vancomycin MIC < 1.5 µg/mL n=28	Vancomycin MIC ≥ 1.5 µg/mL n=34	P
Mean age (SD)	61.1 (18.5)	60.1 (14.3)	0.396
Male gender (%)	19 (68%)	27 (79%)	0.386
Type of endocarditis:			0.548
▪ Native valve	23 (82%)	25 (74%)	
▪ Prosthetic valve	5 (19%)	9 (27%)	
Origin of acquisition:			0.916
▪ Community-acquired	17 (61%)	23 (68%)	
▪ Nosocomial	8 (29%)	9 (26%)	
▪ Health-care related	2 (7%)	2 (6%)	
▪ Unknown	1 (3%)	0	
Geographical area:			0.333
▪ North America	4 (14%)	10 (29%)	
▪ Europe/Mid East	20 (71%)	22 (65%)	
▪ South America	1 (4%)	0	
▪ Australia/New Zealand	3 (11%)	2 (6%)	
Complications:			
▪ Heart failure	13 (46%)	10 (29%)	0.195
▪ Systemic emboli	10 (36%)	8 (23%)	0.400
▪ Stroke	7 (25%)	9 (27%)	1.000
▪ Paravalvular complications	8 (29%)	11 (32%)	0.788
▪ New conduction abnormality	0 (-)	3 (9%)	0.245
▪ Persistent bacteremia	4 (14%)	4 (12%)	1.000
Surgical treatment	9 (32%)	14 (41%)	0.599
Relapses*	0	0	1.000

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Mortality			
▪ In-hospital mortality	9 (32%)	9 (27%)	0.780
▪ One-year mortality	12 (43%)	10 (29%)	0.298

* Defined as a new episode of endocarditis due to the same microorganism that caused the first IE within the following 12 months.

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Table 2. Bi-variable Analysis of Risk Factors for One-year Mortality

Variable	Alive n=39	Dead n=22	OR (95%CI)	<i>P</i>
Demographics				
Age >65years	16 (40%)	11 (50%)	1.5 (0.5-4.9)	0.593
Male sex	31 (78%)	15 (68%)	0.62 (0.2-2.4)	0.546
Prosthetic valve IE	11 (29%)	3 (14%)	0.41 (0.1-1.9)	0.338
Diabetes	8 (20%)	4 (18%)	0.89 (0.2-3.9)	1.000
Acquisition in the community	26 (65%)	14 (64%)	1.76 (0.0-141.9)	0.889
North America	8 (20%)	6 (27%)	1.77 (0.1-143.1)	0.444
Clinical features				
Paravalvular complications	8 (20%)	11 (50%)	4.0 (1.1-14.6)	0.021
Stroke	6 (15%)	10 (46%)	4.72 (1.2-19.1)	0.015
Heart failure	10 (46%)	10 (46%)	1.73 (0.5-5.7)	0.411
Persistent bacteremia	5 (13%)	3 (14%)	1.11 (0.2-6.4)	1.000
Surgery	14 (35%)	9 (41%)	1.29 (0.4-4.2)	0.785
Microbiological features				
Vancomycin MIC \geq 1.5 mg/L	24 (60%)	10 (45%)	0.56 (0.2-1.8)	0.298
CC30	7 (18%)	6 (27%)	1.77 (0.4-7.3)	0.516
CC8	1 (3%)	3 (14%)	6.16 (0.4-331.3)	0.546
CC15	6 (15%)	0	0.0 (0-1.1)	0.081
AGR subgroup I	15 (38%)	7 (32%)	0.78 (0.2-2.6)	0.784
AGR subgroup II	13 (33%)	6 (27%)	0.78 (0.2-2.7)	0.777
AGR subgroup III	11 (28%)	9 (41%)	1.83 (0.5-6.2)	0.394
<i>see</i>	8 (20%)	10 (46%)	3.33 (0.9-12.2)	0.044
<i>sei</i>	33 (83%)	22 (100%)	2.94 (1.1-13.7)	0.044
<i>chp</i>	39 (98)	16 (73%)	0.07 (0.0-0.7)	0.006
<i>eta</i>	8 (20%)	6 (27%)	1.5 (0.4-5.9)	0.539
<i>pvl</i>	4 (10%)	5 (23%)	2.65 (0.5-14.9)	0.259

The analysis for in-hospital mortality using the same variables did not differ from that of one-year mortality (data not shown).

Table 3. Summary of Main Findings from Studies Assessing the Relationship between Vancomycin MIC and Prognosis in MSSA Bacteremia and Left-sided IE.

First author, year	Design	SAB/IE (n)	Overall mortality Low VAN MIC < 1.5 µg/mL	Overall mortality High VAN MIC ≥ 1.5 µg/mL	Genetic factors	Main outcomes analysis
Kalil, 2014 [15]	Systematic review and metaanalysis	SAB (8291, both MRSA and MSSA)	25.8% (1430/5551)	26.8% (734/2740)	NA	RD 1.6% [95%CI, -2.3% to 5.6%]; P = 0.43 (for absolute risk of mortality, combining 30-day mortality and in-hospital mortality)
Holmes, 2011 [1]	Prospective multicenter cohort study	SAB (532; 266 of which MSSA treated with betalactams only)	12.2% (24/193)	26.8% (18/68)	NA	P= 0.011 (for 30-day mortality)
Holmes, 2014 [11]	Analysis of a subset of strains from [1]	SAB (252 MSSA isolates)	NA	NA	Associated to HVM: CC8, <i>agr</i> dysfunction, <i>agr</i> genotype II, <i>blaZ</i> , <i>sea</i> , <i>clfA</i> , <i>sp/A</i> and ACME locus. Associated to LVM: CC22, CC88 and CC188	Associated to HVM: CC8 P < 0.001), <i>agr</i> dysfunction (P= 0.014), <i>agr</i> genotype II (P=0.043), <i>blaZ</i> (P=0.002), <i>sea</i> (P < 0.001), <i>clfA</i> (P < 0.001), <i>sp/A</i> (P 0.001) and ACME locus (P=0.02). Associated to LVM: CC22 (P < 0.001), CC88 ([P < 0.001) and CC188 (P=0.002)
Aguado, 2011 [2]	Retrospective, single-center Cohort	Catheter-related SAB (99, all MSSA)	10.5% (8/76)	26.1% (6/23)	NA	P=0.13 (for 30-day mortality) OR=22.9, [95%CI 6.7–78.1] for complicated SAB.

López-Cortes, 2015 [4]	Prospective, single-center cohort	SAB (135, all MSSA)	23.6% (25/106)	10.3% (3/29)	There were no differences in <i>agr</i> distribution or absence of δ -haemolysin between isolates with HVM and those with LVM. HVM was not more frequent in specific clones.	RR=0.44 [95%CI, 0.14-1.35] for 14-day mortality
Viedma, 2014 [3]	Retrospective, single-center cohort	SAB (84, all MSSA)	24.1%, (7/29)	45.5% (25/55)	HVM: <i>agr II</i> polymorphism: 17.2%; average levels of RNAIII gene expression: Δ Ct 1.5 \pm 2.11 LVM: <i>agr II</i> polymorphism: 41.8%; average levels of RNAIII gene expression: Δ Ct 4.05 \pm 3.29	In-hospital mortality: P=0.057 <i>agr</i> dysfunction: P=0.023. RNAIII expression: P<.01
Cervera, 2014 [5]	Prospective, single-center cohort	MSSA IE (93)	31% (16/53)	53% (21/40)	NA	In-hospital mortality: P=0.035; Patients with HVM presented significantly more severe embolic events
Current study	Prospective, multicenter cohort	MSSA IE (62)	32% (9/28)	27% (9/34)	HVM: <i>agrII</i> polymorphism: 19% LVM: <i>agrII</i> polymorphism: 38%	In-hospital mortality: P=0.780. <i>agrII</i> polymorphism: P=0.157

CI: Confidence interval; IE: Infective endocarditis; MRSA: methicillin-resistant *Staphylococcus aureus*; NA: not addressed; OR: Odds ratio; RD: relative difference; RR: relative risk; SAB: *S. aureus* bacteremia.

Supplement Appendix

1. Microbiological Methods

E-test for Vancomycin MIC

MSSA strains were stored at -80° C in a central laboratory (Duke University) and tested for vancomycin MIC by E-test after the freezing of the samples, following the manufacturer's recommendations (bioMérieux, Marcy l'Etoile, France). E-test were done by duplicate and interpreted by consensus with a second researcher.

Multiplex Polymerase Chain Reaction. Genomic DNA was prepared as previously described [1]. Bacterial determinants including adhesins, toxins, *agr* group I–IV, and other genes were screened by multiplex polymerase chain reaction (PCR) as described before [1]. All negative calls on the multiplex PCR were confirmed by uniplex PCR.

Spa Typing. Spa typing was performed as previously described [1, 2]. PCR oligonucleotide primers for *spa* were described previously [2]. Samples were sequenced at the Duke University sequencing laboratory. For *spa* typing, eGenomics software (<http://tools.egenomics.com/>) was used to scan the primary sequence to help identify the orders and names of each repeat. The *spa* type number is representative of the repeat organization. Clonal complexes (CC) for the isolates were identified via repeats pattern recognition from 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 typing. For MLST, the sequence chromatograms for unique alleles were deposited in the MLST database (<http://www.mlst.net>). Alleles at the 7 loci

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(*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 6 of 7 alleles by using the eBURST algorithm (<http://eburst.mlst.net>) [3].

References

- [1] Campbell SJ, Deshmukh HS, Nelson CL, Bae IG, Stryjewski ME, Federspiel JJ, et al. Genotypic characteristics of *Staphylococcus aureus* isolates from a multinational trial of complicated skin and skin structure infections. *J Clin Microbiol.* 2008;46:678-84.
- [2] Fowler VG Jr, Nelson CL, McIntyre LM, Kreiswirth BN, Monk A, Archer GL, et al. Potential associations between hematogenous complications and bacterial genotype in *Staphylococcus aureus* infection. *J Infect Dis.* 2007;196:738-47.
- [3] Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol.* 2004;186:1518-30.

2. Genotypic characteristics of MSSA strains according to VAN MIC.

Differences in genotypic characteristics between the two groups according to MIC phenotypes are displayed in the **Table 1**. No differences were detected regarding adhesins, toxins or other putative virulence factors. Thirty-eight percent of patients with HVM presented *agr*-II compared to 19% of patients with LVM. However, this difference was not statistically significant ($P=0.157$). With regard to CC, CC30 was the most frequent CC in the whole cohort. Differences

51 in the prevalence of the CCs between the HVM and LVM groups were not
52 statistically significant.

53 **Table 1. Genotypic Characteristics and Distribution of Clonal Complexes**
54 **of 62 Episodes of Left-sided Methicillin-susceptible *Staphylococcus***
55 ***aureus* Infective Endocarditis Treated with Beta-lactams According to**
56 **High or Low Vancomycin Minimum Inhibitory Concentration (MIC).**
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	Vancomycin MIC < 1.5 µg/mL n=28	Vancomycin MIC ≥ 1.5 µg/mL n=34	<i>P</i>
Adhesins			
- <i>fnbA</i>	28 (100%)	34 (100%)	1.000
- <i>fnbB</i>	5 (19%)	7 (21%)	1.000
- <i>clfA</i>	28 (100%)	34 (100%)	1.000
- <i>clfB</i>	28 (100%)	34 (100%)	1.000
- <i>cna</i>	25 (90%)	29 (85%)	0.720
- <i>spa</i>	27 (96%)	33 (97%)	1.000
- <i>scrC</i>	12 (43%)	16 (47%)	0.801
- <i>sdrD</i>	18 (64%)	19 (56%)	0.606
- <i>sdrE</i>	12 (43%)	16 (47%)	0.801
- <i>bbp</i>	25 (89%)	28 (82%)	0.495
- <i>ebpS</i>	28 (100%)	34 (100%)	1.000
- <i>map/eap</i>	17 (61%)	25 (74%)	0.413
Toxins			
- <i>eta</i>	4 (14%)	10 (29%)	0.225
- <i>etb</i>	1 (4%)	1 (3%)	1.000
- <i>tst</i>	27 (96%)	31 (91%)	0.620
- <i>sea</i>	21 (75%)	19 (56%)	0.182
- <i>seb</i>	0	2 (6%)	0.497
- <i>sec</i>	6 (21%)	10 (29%)	0.566
- <i>sed</i>	5 (18%)	9 (27%)	0.546