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Original article

Association between biomass formation and the prognosis of infective endocarditis due to *Staphylococcus aureus*

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ABSTRACT

Introduction: The aim of this study was to evaluate the association between biomass formation and the clinical characteristics and prognosis of *Staphylococcus aureus* infective endocarditis (IE).
Methods: We prospectively studied 209 *S. aureus* strains causing IE. Biomass formation was examined using the crystal violet assay and quantified spectrophotometrically. The average (SD) optical density of the biomass was compared for each clinical, microbiological (methicillin-resistance, vancomycin MIC ≥ 1.5 μg/ml) and molecular (clonal complex, *agr* type and *agr* dysfunction) variable according to their presence or absence. The primary clinical endpoints studied were in-hospital death, severe sepsis, persistent bacteraemia, symptomatic peripheral embolisms and prosthetic valve IE.
Results: Mean age was 66.1 years, 61.5% of patients were male and the median age-adjusted Charlson comorbidity index was 5 points (IQR 3–8). In-hospital mortality was 37.3%. Strains belonging to CC5 and CC22 had optical biomass densities [mean (SD) 1.573 (1.14) vs 0.942 (0.98) $p < 0.001$ and 1.720 (0.94) vs 1.028 (1.04) $p = 0.001$, respectively]. Strains belonging to CC5 and CC22 had significantly higher optical biomass densities [1.369 (1.18) vs 0.920 (0.93) $p = 0.008$]. No statistically significant differences were found in the clinical endpoints studied.
Conclusions: High biomass production was associated with CC5 and CC22 but not with higher hospital mortality, septic complications, type of endocarditis, methicillin-resistance, elevated vancomycin MIC or *agr* dysfunction.

Asociación entre la formación de biomasa y el pronóstico de la endocarditis infecciosa causada por *Staphylococcus aureus*

Palabras clave:
Staphylococcus aureus
Endocarditis infecciosa
Biopelícula
Biomasa
Densidad óptica
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RESUMEN

Introducción: La bacteriemia por *Staphylococcus aureus* es un problema de salud importante asociado a una elevada mortalidad. El objetivo de este estudio fue evaluar la asociación entre la capacidad de formación de biomasa y las características clínicas y el pronóstico de la endocarditis infecciosa (EI) por *Staphylococcus aureus*.
Métodos: Se estudiaron de forma prospectiva 209 cepas de *S. aureus* causantes de episodios de EI. La formación de biomasa se estudió mediante la técnica de cristal violeta y se cuantificó por espectrometría. La media (DE) de la densidad óptica de la biomasa se comparó para cada variable clínica, microbiológica (resistencia a la meticilina, CMI de vancomicina ≥ 1,5 μg/ml) y molecular (complejo clonal, tipo y

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disfunción de *agr*) según su presencia o ausencia. El criterio principal de valoración fue la mortalidad hospitalaria. Otras variables clínicas evaluadas fueron: septicemia grave, bacteriemia persistente, embolias periféricas sintomáticas y EI sobre válvula protésica.
Resultados: La edad media (DE) fue de 66,1 (16,2) años, el 61,5% eran varones y la mediana del índice de comorbilidad de Charlson ajustado a la edad fue de 5 puntos (RIC 3-8). La mortalidad hospitalaria fue del 37,3%. Las cepas pertenecientes a CC5 y CC22 presentaron densidades ópticas de biomasa significativamente más elevadas (media [DE] 1,573 [1,14] frente a 0,942 [0,98] p < 0,001 y 1,720 [0,94] frente a 1,028 [1,04]; p = 0,001, respectivamente). Las cepas pertenecientes a los grupos *agr*II mostraron mayores densidades ópticas de biomasa (1,369 [1,18] frente a 0,920 [0,93]; p = 0,008). No se observaron diferencias estadísticamente significativas en las variables clínicas estudiadas.
Conclusiones: La producción elevada de biomasa se asoció a determinados linajes clonales (CC5 y CC22), pero no se asoció a una mayor mortalidad hospitalaria, complicaciones sépticas, tipo de endocarditis, resistencia a la meticilina, CMI de vancomicina elevada o disfunción del *agr*.

Introduction

Infective endocarditis (IE) is an uncommon and severe disease with in-hospital mortality around 20%,[1] being greater than 30% in infections due to *Staphylococcus aureus*.[2] The poor prognosis of *S. aureus* bacteremia is conditioned by factors associated with the host, the management of infection, and the intrinsic characteristics of the bacteria. Among the latter, there is a great need to determine whether factors other than antimicrobial resistance that are related to the prognosis can assist with decision making. In this sense, the production of biofilms is considered a variable that is associated with a worse prognosis although scientific evidence is scarce.

Recently, our group published a prospective study of 213 consecutive episodes of *S. aureus* IE with the aim of evaluating the impact of the phenotype and genotype on the clinical characteristics and prognoses of these patients.[2] However, despite a recent study of 485 episodes of *S. aureus* bacteremia from different sources of infection found no relationship between biofilm production and a worse prognosis (30-day related mortality, infective endocarditis (IE), persistent bacteremia or recurrent bacteremia),[3] we aimed to analyze the relationship between the biomass formation capacity and the clinical characteristics and prognosis of 209 consecutive episodes of *S. aureus* IE.

Methods

A detailed description of the study design can be found elsewhere.[2] Briefly, a multicenter, longitudinal, prospective, observational study was performed in 15 hospitals with broad experience in IE. Between June 2013 and March 2016, consecutive adult patients (≥18 years) with a definite diagnosis of *S. aureus* IE were enrolled in the study.

Clinical, microbiological and prognostic variables were collected. The primary outcome was in-hospital death. Other clinical variables were healthcare-associated acquisition, previous use of antibiotics, severe sepsis/septic shock, persistent bacteremia 3 days after starting antimicrobial therapy, persistent bacteremia 5 days after starting antimicrobial therapy, symptomatic peripheral embolism (any), symptomatic osteoarticular involvement, native valve only IE, prosthetic valve IE, intracardiac device IE, surgery indicated and performed or indicated but nor performed, and a composite end-point including persistent bacteremia 3 or 5 days after starting antimicrobial therapy plus symptomatic peripheral embolism (any).

In-hospital death was defined as all-cause death during the hospital stay. For this study, persistent bacteremia was registered as a categorical variable (demonstration of positive blood cultures ≥3 and ≥5 days after initiation of active antimicrobial therapy).

The first isolate from each patient was included. Blood cultures were processed and bacterial identification and antimicrobial susceptibility testing were performed according to standard techniques. Vancomycin MICs were determined by the E-test method. Bacterial DNA was extracted using commercial extraction kits (Qiagen, Germany) according to the manufacturer's recommendations. DNA microarrays (Alere, Germany)[4] covering species-specific markers, clonal complex (CC) and *agr* group typing markers were run for the entire collection of strains.

The functionality of the *agr* operon was measured by δ-hemolysin production as determined by streaking each *S. aureus* IE isolate adjacent to *S. aureus* strain RN4220 on a trypticase soy agar plate supplemented with 5% sheep blood and incubating overnight at 37 °C.[5]

Biomass production by the bacterial strains was assessed according to the protocol described by Stepanović et al.[6] with slight modifications. Briefly, biofilm growth was set up in 96-well polystyrene plates with the bacterial suspension (200 μL containing 1.0×10^6 colony-forming units/mL) in trypticase soy broth (TSB, Becton Dickinson and Company, Le Pont de Claix, France) supplemented with 1% glucose (Sigma-Aldrich Co., Madrid, Spain). The plates were incubated for 24 h at 37 °C under static conditions. Following incubation, the supernatant was discarded, and the wells were washed twice with distilled water. The plates were air-dried for 1 h at 37 °C and stained with 200 μL/well of 0.1% crystal violet solution (Sigma-Aldrich Co., Madrid, Spain) at room temperature for 15 min. The plates were washed twice with distilled water, and 200 μL of 33% glacial acetic acid (VWR International Eurolab, Barcelona, Spain) was added to each well. The optical density (OD) was measured at 590 nm using a microplate spectrophotometer (ELx800, Biotek, VT, USA). The assay was performed in triplicate.

The average optical density of the biomass was compared for each clinical, microbiological and molecular variable according to their presence or absence, using the Mann–Whitney test. Statistical significance was set at $p < 0.05$, and the hypotheses were two-sided.

This study was approved by the Ethics Committee of each participating hospital and the Spanish Drug Agency (IRH-ANT 2013-01).

Results

The original study included 213 consecutive episodes of definitive *S. aureus* IE [mean age 66.1 years (SD 16.2), 61.5% male, median age-adjusted Charlson comorbidity index 5 points (IQR 3–8)]. In four of these episodes, the CC of the strain causing the infection

Table 1
Comparison of the mean (SD) optical density of biomass according to the presence or absence of clinical, microbiological and molecular variables in 209 strains of *S. aureus* causing definite infective endocarditis.

Variable	*N* (%)	Presence [mean (SD) of OD biomass]	Absence [mean (SD) of OD biomass]	*p*-Value
Clinical characteristics				
Healthcare-associated IE acquisition	111 (53.1)	1.074 (1.06)	1.089 (1.04)	0.918
Previous use of antibiotics (1 month)	49 (23.4)	1.004 (1.06)	1.105 (1.05)	0.562
Severe sepsis/septic shock	95 (45.4)	0.999 (1.02)	1.180 (1.08)	0.250
Persistent bacteremia at day 3	60 (28.7)	1.167 (1.15)	1.046 (1.01)	0.966
Persistent bacteremia at day 5	36 (17.2)	1.071 (1.07)	1.083 (1.05)	0.716
Symptomatic peripheral embolism	103 (49.3)	1.119 (1.07)	1.044 (1.02)	0.686
Osteoarticular metastasis	25 (12.0)	1.389 (1.32)	1.039 (1.00)	0.449
Native valve endocarditis only	143 (68.4)	1.555 (1.10)	0.930 (0.91)	0.444
Prosthetic valve endocarditis	39 (18.7)	0.982 (0.99)	1.104 (1.06)	0.496
Intracardiac device IE	27 (12.9)	0.831 (0.78)	1.118 (1.08)	0.098
Complications at day 3[†]	127 (60.8)	1.070 (1.04)	1.054 (1.06)	0.848
Complications at day 5[††]	117 (56.0)	1.102 (1.05)	1.054 (1.05)	0.657
Surgery indicated and performed	79 (37.8)	1.052 (0.91)	1.217 (0.91)	0.332
Surgery indicated but not performed	54 (25.8)	0.932 (1.02)	1.133 (1.05)	0.220
In-hospital mortality	78 (37.3)	1.244 (1.20)	0.984 (0.93)	0.425
Clonal complexes (CC)				
CC5	46 (22.0)	1.573 (1.14)	0.942 (0.98)	<0.001
CC8	23 (11.0)	0.328 (0.25)	1.174 (1.07)	<0.001
CC15	19 (9.1)	0.575 (0.84)	1.132 (1.05)	0.002
CC22	16 (7.7)	1.720 (0.94)	1.028 (1.04)	0.001
CC30	40 (19.1)	0.790 (0.79)	1.150 (1.09)	0.062
CC45	18 (8.6)	0.322 (0.14)	1.152 (1.07)	<0.001
agr type				
*agr*I	79 (37.8)	0.927 (0.93)	1.175 (1.10)	0.187
*agr*II	75 (35.9)	1.369 (1.18)	0.920 (0.93)	0.008
*agr*III	45 (21.5)	0.841 (0.84)	1.147 (1.09)	0.123
*agr*IV	–	–	–	–
agr dysfunction (δ-hemolysin)	153 (73.2)	1.025 (1.03)	1.235 (1.09)	0.105
Methicillin-resistant *S. aureus*	41 (19.6)	1.365 (1.24)	1.014 (0.99)	0.180
Vancomycin MIC ≥1.5 μg/ml	74 (35.4)	0.961 (1.02)	1.147 (1.06)	0.195

Data are expressed as the mean (standard deviation). IE: infective endocarditis.
[†] Composite end-point including persistent bacteremia 3 days after starting antimicrobial therapy plus symptomatic peripheral embolism (any).
[††] Composite end-point including persistent bacteremia 5 days after starting antimicrobial therapy plus symptomatic peripheral embolism (any).

could not be determined. Thus, we used 209 strains in the present study.

In the subgroups of patients analyzed, 111 (53.1%) infections were healthcare-associated, 143 (68.4%) affected native valves only, 39 (18.7) prosthetic valves, and 27 (12.9%) intracardiac devices. In 41 (19.6%) the infection was caused by a methicillin-resistant strain. Severe sepsis/septic shock was present in 95 (45.4%). After starting active treatment, persistent bacteremia at day 3 was demonstrated in 60 (28.7%) and at day 5 in 36 (17.2%). Symptomatic peripheral embolism was present in 103 (49.3%) and symptomatic osteoarticular metastasis in 25 (12.0%). Surgery was indicated in 133 (63.6%) but performed only in 79 (37.8%). In-hospital mortality was 37.3%.

Table 1 compares the mean (SD) optical densities of biomass for the 209 *S. aureus* strains included in the study according to the presence or absence of clinical, microbiological and molecular variables. No statistically significant differences were found in the optical densities [OD (SD)] of the biomass between strains causing IE with and without clinical complications, including in-hospital death [1.244 (1.20) vs 0.984 (0.93), $p = 0.425$]. Additionally, no statistically significant differences were observed in the optical densities of the biomass according to the type of endocarditis [native valve only 1.555 (1.10) vs 0.930 (0.91), $p = 0.444$; prosthetic valve 0.982 (0.99) vs 1.104 (1.06), $p = 0.496$; intracardiac device 0.831 (0.78) vs 1.118 (1.08), $p = 0.098$], healthcare-associated infection [1.074 (1.06) vs 1.089 (1.04), $p = 0.918$] or previous use of antibiotics [1.004 (1.06) vs 1.105 (1.05), $p = 0.562$]. Strains belonging to CC5 and CC22 had significantly higher biomass optical densities [1.573 (1.14) vs 0.942 (0.98), $p = <0.001$ and 1.720 (0.94) vs 1.028 (1.04), $p = 0.001$, respectively], which was in contrast to the strains belonging to CC8, CC15 and CC45, which had significantly lower optical densities [0.328 (0.25) vs 1.174 (1.07), $p < 0.001$; 0.575 (0.84) vs 1.132 (1.05), $p = 0.002$; and 0.322 (0.14) vs 1.152 (1.07), $p < 0.001$, respectively]. The strains belonging to the *agr*II group showed a biomass optical density that was significantly higher than that of the rest of the *agr* groups [1.369 (1.18) vs 0.920 (0.93), $p = 0.008$]. Finally, no differences were found in *agr* dysfunction, methicillin resistance or vancomycin MICs.

Discussion

In this study, high biomass production was not associated with higher crude in-hospital mortality, septic complications, type of endocarditis, healthcare-associated infections or previous use of antibiotics. Moreover, it was also not associated with methicillin resistance, elevated vancomycin MICs, or *agr* dysfunction. However, strains belonging to the clonal complexes CC5 and CC22 showed high biomass production, which was in contrast to the strains belonging to CC8, CC15 and CC45, which were related to lower biomass production. Finally, strains belonging to the *agr*II group showed a biomass optical density that was significantly higher than that of the rest of the *agr* groups.

Classically, the biofilm production capacity has been considered a key factor that explains the morbidity and mortality of infections caused by *S. aureus*.[7] However, this hypothesis has not been proven through clinical studies.

S. aureus bacteremia is not a homogeneous entity but instead is a sign of the spread of an infection that can originate in very diverse locations. Unlike the study of Guembe et al.,[3] which included bacteremia due to *S. aureus* of any origin and in which no subgroups

were analyzed, our study included only *S. aureus* strains causing definite episodes of IE. However, our study confirms the hypothesis that a high biomass production capacity is not related to a worse prognosis.

In the present study, CC5 and CC22 were the clonal complexes related to higher biomass production. However, none of these complexes was associated with mortality or complications in the previous clinical study.[2] These results are consistent with those of another study of clinically invasive isolates, in which CC5 proved to be a strong biofilm producer.[8] Conversely, CC8 and CC15, which were clonal complexes that produced fewer biomass, were respectively associated with higher in-hospital mortality and higher early mortality (≤2 days). Finally, CC45, which was the other clonal complex that produced fewer biomass, was related to infections on prosthetic valves and intracardiac devices.[2]

S. aureus infective endocarditis is an acute infection.[2] This characteristic could justify the absence of a clinical influence of biomass production showed in this study. Whatever the reason, our results are consistent with those of a recent study conducted on 159 *S. aureus* strains causing invasive infections, in which the strains causing endocarditis did not show a greater capacity for biofilm formation nor the production of biofilm was associated to complicated bacteremia, severe sepsis, 28-day mortality or recurrence.[9]

No study is exempt from limitations. First, although the crystal violet assay is the most widely used method to quantify biomass production, we did not evaluate the metabolic activity of biofilm by means of XTT assay.[10] Moreover, features such as host blood flow, serum proteins and other components of the immune system are not accurately reflected in the in vitro model. Second, we only measured biomass production by the first isolate from each episode; therefore, we do not know whether biomass production would have occurred in the strains of persistent isolates. Third, the correlation between the phenotypic test for the detection of *agr* operon dysfunction and its expression is not always good, and therefore the most appropriate technique would be quantification of RNAIII expression (gene effector of the *agr* operon).[11,12]

In conclusion, in this study high biomass production was associated with some clonal lineages (CC5 and CC22) but was not associated with higher hospital mortality, septic complications or other predisposing factors.

Investigators

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Conflict of interest

All authors declare that they have no conflicts of interest.

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