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Enhancement of antistaphylococcal activities of six antimicrobials against *sas*G-negative methicillin-susceptible *Staphylococcus aureus*: an in vitro biofilm model☆

Keli Cristine Reiter [a,b,*], Bárbara Villa [b], Thiago Galvão da Silva Paim [a,b], Gustavo Enck Sambrano [b], Caio Fernando de Oliveira [a,b], Pedro Alves d'Azevedo [a,b]

[a] Health Sciences Post-Graduate Program, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil
[b] Laboratory of Gram-Positive Cocci, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil

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ABSTRACT

This study was designed to evaluate antimicrobial activities against methicillin-susceptible *Staphylococcus aureus* in both sessile and planktonic forms and to detect genes associated with this biofilm phenotype. Minimal biofilm inhibition and eradication concentrations (MBIC and MBEC, respectively) were determined by an in vitro biofilm model, and *ica*A, *atl*A, and *sas*G genes were detected by polymerase chain reaction. Vancomycin and tigecycline presented better biofilm inhibitory activity (MBIC range: 4–8 μg/mL) ($P \leq 0.05$) and lower MBEC/MIC ratios ($P \leq 0.001$) than other antimicrobials. All isolates harbored *ica*A and *atl*A, whereas *sas*G was present only in strong biofilm formers ($P \leq 0.05$). Interestingly, antimicrobial activities against *sas*G− weak biofilm formers were significantly higher than those against *sas*G+ strong biofilm formers ($P \leq 0.05$), demonstrating that number of cells in a biofilm matrix affected the antimicrobial activity, which was also variable, and might be associated with specific genetic determinants. To our knowledge, this was the first study reporting the presence of *sas*G in clinical isolates of *S. aureus* in South America.

1. Introduction

Bacterial adherence to implanted or indwelling devices, bone or natural tissue, and other hydrophobic surfaces is the leading virulence factor in staphylococci. These microorganisms are extensively known for their ability to grow as a bacterial cell community, be embedded in biofilm, exchange essential nutrients, and for their mobile genetic materials (Götz, 2002).

In biofilm, bacteria encase themselves in an extracellular material (slime), which embeds them together and attaches them firmly to a surface. Biofilm formation is a multistep virulence process that initiates with attachment mediated by specific proteins called autolysins, one of which is known as AtlA in *Staphylococcus aureus*, encoded by the *atl*A gene (Biswas et al., 2006). Furthermore, intercellular adhesion occurs due to production of a polysaccharide intercellular adhesin (PIA) encoded by the *ica*ADBC locus (*ica*-dependent pathway) or due to other proteins involved in this accumulation phase (*ica*-independent pathway) such as a surface protein called SasG (Heilmann et al., 1996; Corrigan et al., 2007; Geoghegan et al., 2010; Montanaro et al., 2011). Autolysins are often produced throughout the growth cycle and have been shown to play a central role in other functions such as cell growth, cell-to-surface adhesion, genetic competence, and pathogenicity (Heilmann et al., 1997; Smith et al., 2000; Takahashi et al., 2002). Maybe, its major role in the process of biofilm formation is to orientate and expose cell-surface adhesins correctly to maximize and ensure effective interaction with biotic or abiotic surfaces (Stevens et al., 2009).

Biofilm-related infections are particularly serious in patients with indwelling medical devices, since cells or clusters of cells may detach from this mucoid slime, resulting in bloodstream infection, emboli, and metastatic spread. Treatment of these infections is increasingly problematic because cells embedded in biofilms are inherently resistant to host immune responses and antimicrobial chemotherapy (Fitzpatrick et al., 2005). Vancomycin is the preferred treatment for *S. aureus* infections (Michel and Gutmann, 1997), and divergent prevalence of this microorganism around the world could guide the antimicrobial therapy to another course, mainly due to increasing rates of vancomycin failure on *S. aureus* infection therapy (Hidayat et al., 2006; Neoh et al., 2007; Hsu et al., 2008). In addition, knowledge of specific antimicrobial activity against biofilm-forming staphylococci is an important determinant for choosing preventive or curative antimicrobial therapy, as well as MIC measurement against sessile cells (cells embedded in biofilm). Even if a biofilm-related infection

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* Corresponding author. Tel.: +55-51-33038840; fax: +55-51-33038810.
E-mail addresses: kelicrisr@gmail.com, kelir@ufcspa.edu.br (K.C. Reiter).

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seems to be cured by antimicrobial therapy, a subset of bacteria can survive within the remaining biofilm and then the infection persists (Costerton et al., 1999; Mah and O'Toole, 2001). Consequently, device-related biofilm-associated infections usually require device removal besides systemic antimicrobial therapy. However, access vein loss, device replacement, and the high cost of this procedure call for saving the infected device when the clinical situation allows it (Mermel et al., 2001).

The aim of this study was to evaluate erythromycin, gentamicin, oxacillin, rifampicin, tigecycline, and vancomycin activities against methicillin-sensitive *S. aureus* (MSSA) in both sessile and planktonic forms and determine the frequency of specific genes associated with biofilm phenotype.

2. Materials and methods

2.1. Bacterial isolates

Fifteen known biofilm-forming MSSA obtained from different patients with catheter-related bloodstream infections at Complexo Hospitalar Santa Casa de Misericordia de Porto Alegre (Porto Alegre, Brazil) were evaluated. They were selected between August and December 2010 and previously studied according to general characteristics such as resistance profile, presence of *mec*A gene, and ability to produce biofilm. All MSSA strains were *mec*A negative and capable of biofilm formation, and they seemed to be more prevalent in our setting, so they warrant attention in this study (Reiter et al., 2011).

2.2. Biofilm phenotypic assay

Biofilm production was determined by microtiter plate assay, and optical density results were scored and interpreted as described elsewhere (Stepanović et al., 2007; Reiter et al., 2011). Briefly, 180 μL of trypticase soya broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with glucose 1% was added to each well of a sterile 96-well polystyrene flat-bottom microtiter plate (TPP Techno Plastic Products, Trasadingen, Switzerland), followed by 20 μL of 1×10^8 CFU/mL bacterial suspension (1:10 dilution). The plates were incubated for 24 h at 35 ± 2 °C under static conditions. After incubation and broth removal, wells were washed 3 times with sterile saline and bacteria attached were fixed with methanol for 20 min and left to air dry overnight in an inverted position at room temperature. Finally, adherent bacteria were stained with crystal violet 0.5% for 15 min and biofilm was eluted with ethanol for 30 min without shaking. Absorbance was measured at 492 nm using the microtiter plate reader Expert Plus (ASYS Hitech, Eugendorf, Austria).

The cut-off value (ODc; optical density of negative control at 492 nm) was defined as 3-fold the standard deviation (SD) above negative control (in practical terms, a reading around 0.090 at 492 nm), and isolates were categorized into strong ($2ODc \leq OD \leq 4ODc$) and weak ($ODc < OD < 2ODc$) biofilm formers.

2.3. Biofilm genotypic assay

Genotypic characteristics were determined by polymerase chain reaction (PCR) and negative results were performed in duplicate. The genes *ica*A (Abraham and Jefferson, 2010), *atl*A (Wootton et al., 2005), and *sas*G (Abraham and Jefferson, 2010) were evaluated, and the fragments were compared to a molecular weight pattern, respectively, as follows: icaA-F 5′-AAACTTG GTGCGGTTACAGG-3′, icaA-R 5′-GTAGCCAACGTCGACAACTG-3′ (188 bp), atlA-F 5′-CAGTTAGCAA-GATTGCTCAAG-3′, atlA-R 5′-CCGTTACCTGTTTCTAATAGG-3′ (1035 bp), and sasG-F 5′-ACCACAGGGTGTAGAAGCTAAATC-3′, sasG-R 5′-CGAGC TTTTCTAA CCTTAGGTGTC-3′ (188 bp). All 3 primer pairs were confirmed as amplifying conserved regions of their correspondent genes. Investigation of strain variation and B subunit repeats of *sas*G was not performed, since the primer pair annealing occurs upstream of these repeats in the gene conserved region.

PCR was performed for each gene alone. Briefly, 1 μL of bacterial DNA (10 μg/mL) was added to 24 μL of PCR mixture containing Tris–HCl buffer (pH 8.4), 1.5 μmol/L of $MgCl_2$, 0.25 mmol/L of each deoxynucleotide triphosphate (Invitrogen, Carlsbad, CA, USA), 1.25 U of Platinum Taq DNA polymerase (Invitrogen), and 0.4 μmol/L of a specific primer (Invitrogen).

Amplification was performed in a LifePro Thermal Cycler (Hangzhou Bioer Technology, Hangzhou, China) beginning with an initial denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, ending with a final extension step at 72 °C for 5 min. PCR products were detected on a 1.5% agarose gel and stained with ethidium bromide.

2.4. Antimicrobials

Tigecycline, vancomycin, rifampicin, erythromycin, gentamicin, and oxacillin were selected for susceptibility tests. Vancomycin, gentamicin, and rifampicin are the most recommended therapeutic choices for staphylococcal biofilm-related infection treatment, mainly when they are used in combination (Olson et al., 2010; McConeghy and LaPlante, 2010). Erythromycin and oxacillin are the most prescribed agents in our setting, mostly for multisusceptible *S. aureus*, and tigecycline is one of the newest drugs available for antimicrobial therapy in our country. Analyses of other antimicrobials were considered unnecessary.

Tigecycline powder was provided by Wyeth Pharmaceuticals (Pearl River, NY, USA); vancomycin, rifampicin, oxacillin, erythromycin, and gentamicin analytical powders were provided by Sigma-Aldrich (St. Louis, MO, USA).

2.5. Planktonic-cell susceptibility tests

Each antimicrobial conventional MIC and minimal bactericidal concentration (MBC) was determined by twofold serial broth microdilution according to CLSI (2009). *S. aureus* ATCC 29213 was tested as quality control.

2.6. Sessile-cell susceptibility tests

Minimal biofilm inhibition and eradication concentration (MBIC and MBEC, respectively) experiments were performed as described elsewhere (Labthavikul et al., 2003; Cafiso et al., 2010), with minor modifications. In brief, 20 μL of 108 CFU/mL bacterial suspensions was added to 180 μL of trypticase soy broth supplemented with 1% glucose (final bacterial concentration = 107 CFU/mL) placed into a sterile 96-well polystyrene flat-bottom microtiter plate (TPP Techno Plastic Products, Trasadingen, Switzerland) and incubated for 24 h at 35 °C without shaking, to allow bacterial attachment. Nonadherent cells were removed by gentle washing 3 times with sterile saline. Serial twofold dilutions of each antimicrobial agent in cation-adjusted Mueller-Hinton broth (CAMHB) were added to wells containing adherent cells, and microplates were incubated at 35 °C for another 24 h. MBIC was defined as the minimal antimicrobial concentration at which there was no observable bacterial growth in wells containing adherent microcolonies.

After MBIC determination, CAMHB containing antimicrobials was removed and wells were washed twice with sterile saline and replaced with 100 μL of antimicrobial-free CAMHB, followed by incubation for 24 h at 35 °C. MBEC was defined as the minimal antimicrobial concentration at which bacteria fail to regrow after antimicrobial exposure, i.e., the minimal concentration required for eradicating the biofilm. All determinations were performed in duplicate.

Table 1
Susceptibility results for planktonic and sessile MSSA.

	Erythromycin	Gentamicin	Oxacillin	Rifampicin	Tigecycline	Vancomycin
Planktonic cells						
MIC_{50}	0.5	0.125	0.25	<0.03	0.25	1
MIC_{90}	2	0.25	1	<0.03	0.5	1
MIC range	0.5–64	0.125–0.5	0.25–2	<0.03	0.125–0.5	0.5–1
MBC_{50}	4	2	0.5	0.06	1	1
MBC_{90}	16	4	2	0.06	2	2
MBC range	0.5–256	0.25–4	0.5–4	0.06	1–4	1–4
Sessile cells						
$MBIC_{50}$	64	32	16	32	8	8
$MBIC_{90}$	128	64	128	64	16	8
MBIC range[a]	16–>256	8–>256	16–>256	16–64	2–32	4–8
$MBEC_{50}$	128	128	128	64	16	32
$MBEC_{90}$	256	256	256	128	64	128
MBEC range[b]	64–>256	16–>256	64–>256	32–128	8–256	16–128

One-way ANOVA ($P < 0.001$), followed by Bonferroni's post hoc test.
[a] Statistically significant differences: erythromycin × vancomycin ($P = 0.004$), erythromycin × tigecycline ($P = 0.006$), gentamicin × vancomycin ($P = 0.004$), oxacillin × vancomycin ($P = 0.008$), and oxacillin × tigecycline ($P = 0.013$).
[b] Statistically significant differences: erythromycin × vancomycin ($P < 0.001$), erythromycin × tigecycline ($P < 0.001$), gentamicin × vancomycin ($P = 0.049$), gentamicin × tigecycline ($P = 0.013$), oxacillin × vancomycin ($P < 0.001$), oxacillin × tigecycline ($P < 0.001$), rifampicin × erythromycin ($P = 0.003$), and rifampicin × oxacillin ($P = 0.019$).

This method is not indicated to determine faithfully the number of viable cells in biofilm before and after treatment, since colony counting variability may be high. Therefore, some analyses were not able to be performed and we considered it as a limitation of our study.

2.7. Statistical analysis

All statistical tests were performed using the Statistical Package for the Social Sciences (SPSS) software version 16.0 (SPSS, Chicago, IL, USA). Continuous variables were tested for normal distribution by Shapiro–Wilk test, and data were expressed as numbers. Statistical significance for associations between strong and weak biofilm formers according to MBIC, MBEC, and MBEC/MIC ratios, and OD reading according to *sas*G harboring was calculated using Student's *t* test for independent samples, with significant *P* value ≤0.05. Comparisons between antimicrobials groups ($k = 6$) and MBIC, MBEC, and MBEC/MIC ratios were performed using 1-way ANOVA followed by Bonferroni's post hoc test, with significant *P* value ≤0.05.

3. Results

All MSSA strains were previously evaluated for biofilm production. Among them, 11 were classified as strong biofilm formers and 4 were classified as weak biofilm formers. Detection of biofilm genes showed that all harbored the *ica*A and *atl*A genes, whereas *sas*G was present in 11 of 15 isolates—all strong biofilm formers ($P \leq 0.05$).

Antimicrobial susceptibility against planktonic and sessile bacteria is presented in Table 1. All 6 antimicrobials displayed MIC_{50} <1 μg/mL for planktonic cells and rifampicin displayed the lowest MIC_{90} (<0.03 μg/mL). All antimicrobials reached susceptibility breakpoints (MIC range), except for erythromycin ($MBC_{50} = 4$ μg/mL; $MBC_{90} = 16$ μg/mL). The results obtained on planktonic forms showed that all antimicrobials tested were variably bactericidal, with MBC_{90} ranging from 1 dilution higher to ≥3 dilutions higher than MIC_{90} values for bacteriostatic agents.

Vancomycin and tigecycline presented better inhibitory activity for adherent MSSA than others (MBIC ranges: 4–8 and 2–32 μg/mL), despite high MBEC values ($P \leq 0.05$) (Table 1). Likewise, MBEC values also demonstrated significant differences among all antimicrobials (Table 1), emphasizing again the superior activity of vancomycin and tigecycline. However, comparative analysis between vancomycin and tigecycline showed that there was no difference in biofilm inhibition between the 2 antimicrobials nor in biofilm eradication. MBIC and MBEC results for all antimicrobials in general were significantly higher in strong than in weak biofilm-forming MSSA ($P \leq 0.05$).

In order to verify how high were the antimicrobial concentrations when tested against adherent cells in comparison with planktonic cells, MBEC/MIC ratios were determined and analyzed according to biofilm-producing intensity (strong or weak) (Table 2). Except for tigecycline, the MBEC/MIC ratios of other antimicrobials were significantly higher in strong biofilm-forming than in weak biofilm-forming MSSA ($P \leq 0.05$). As rifampicin MIC values were extremely low (all results <0.03 μg/mL), the relation with MBEC resulted in a particularly high rate, statistically different from all others ($P \leq 0.001$). Conversely, vancomycin showed the lowest rates compared with rifampicin, erythromycin, gentamicin, oxacillin, and tigecycline ($P \leq 0.001$).

4. Discussion

The activity of 6 different antimicrobials against planktonic and biofilm embedded cells was studied. Several mechanisms have been discussed on trying to explain antimicrobial resistance of cells in

Table 2
Intensity of biofilm production compared with each antimicrobial MBEC/MIC ratio.

Antimicrobial	MBEC/MIC ratio[a]		*P* value
	Strong/moderate producer	Weak producer	
Erythromycin	1024 (1)–512 (3)–256 (4)–128 (3)	256 (1)–128 (1)–64 (1)–4 (1)	0.040*
Gentamicin	2048 (4)–1024 (3)–512 (2)–256 (2)	512 (1)–256 (2)–128 (1)	0.026*
Oxacillin	1024 (4)–512 (5)–128 (1)–64 (1)	256 (2)–128 (2)	0.022*
Rifampicin	4267 (6)–2133 (5)	1066 (2)–2133 (2)	0.014*
Tigecycline	1024 (1)–512 (1)–256 (1)–128 (2)–64 (3)–32 (3)	128 (1)–64 (3)	0.108
Vancomycin	256 (2)–128 (3)–64 (6)	16 (2)–32 (2)	0.019*

[a] Ratio (number of isolates); strong/moderate ($n = 11$) and weak producer ($n = 4$).
* $P \leq 0.05$ was considered statistically significant (Students' *t* test for independent samples).

biofilm, mainly for those that are susceptible when tested in routine laboratories. Vancomycin and tigecycline showed better inhibitory activities against adherent MSSA than erythromycin, gentamicin, rifampicin, and oxacillin. Depending on biofilm characteristics and antimicrobial agent used to treat the biofilm, different mechanisms could account for this resistance. However, when performing susceptibility tests in vitro, there are some issues that need to be considered, because they may have an important impact on the interpretation of in vivo situation. Stationary-phase cultures result in diminished killing rates to such an extent that the bactericidal effects of some cell-wall active antibacterial agents are eliminated (e.g., against nongrowing or slowly growing phases of *S. aureus*). It is also important to understand that the definition of bacteriostatic or bactericidal activity for an antibacterial agent applies only to the particular organism (or even strain) against which it has been tested under the particular test conditions used (Pankey and Sabath, 2004). Moreover, different species could behave differently in an antimicrobial therapy, as demonstrated by Qu et al. (2009, 2010) for coagulase-negative staphylococci. In their study, vancomycin MBEC had much worst activity than the one demonstrated by our study, well beyond the highest achievable serum concentrations.

In our study, MBEC results showed that stronger biofilm-producing MSSA strains were more resistant to all antimicrobials tested than weaker ones. Likewise, Antunes et al. (2011) also demonstrated that stronger biofilm-producing staphylococci presented higher vancomycin MBEC results than weaker ones. This characteristic may be associated with biofilm architecture, which is known to be incredibly unique and prevents antimicrobial access due to several circumstances (Mah and O'Toole, 2001). For example, eletrostatic interaction of the antimicrobial with biofilm exopolysaccharide matrix and the physical or chemical structure of these exopolysaccharides may delay antimicrobials penetration or even exclude them from the bacterial community (Mah and O'Toole, 2001). It is possibly created by staphylococcal cells in terms of quantity and exopolysaccharide matrix content—strong biofilm producers present higher numbers of bacterial cells, which contributes to improving the design and shape of biofilm arrangement. In fact, thicker biofilms could present a barrier to compound penetration, in addition to other mechanisms such as antimicrobial degradation by enzymes (Anderl et al., 2000) and antimicrobial binding to the biofilm components (Mah and O'Toole, 2001), so the slow penetration through biofilm exopolysaccharide matrix could facilitate this process.

Despite the large number of antimicrobial agents available to treat infections caused by staphylococci, none has been described that totally eradicates staphylococcal biofilms. Some studies have shown that, when an antimicrobial agent is used for exopolysaccharide matrix disruption, the penetration of other antimicrobials into the biofilm could be facilitated (Glansdorp et al., 2008; Hajdu et al., 2009; Smith et al., 2010). However, the necessity to develop methods to treat and prevent biofilm infections has become of increased importance since the last decades (Fitzpatrick et al., 2005) and still remains relevant. Vancomycin and tigecycline were the most active antimicrobials against MSSA. $MBIC_{50}$ for both drugs and $MBIC_{90}$ for vancomycin reached 8 μg/mL, with a range from 4 to 8 μg/mL for vancomycin. These are reachable serum concentrations for bloodstream infections, and in the case of catheter-related ones, vancomycin could be associated with gentamicin or rifampicin to improve their activities, even more due to high MBEC values.

The biofilm-associated genes evaluated in this study are well supported in the literature for their involvement in biofilm formation. Carriage of the *ica* locus is strongly associated with a biofilm-forming capacity in *S. epidermidis* (Fitzpatrick et al., 2002), but the correlation between *ica* and biofilm formation in *S. aureus* is more ambiguous, even though this locus is maintained and expressed in almost all *S. aureus* isolates (Fitzpatrick et al., 2006; O'Neill et al., 2008). The role of the *ica* locus in *S. aureus* is complex, particularly given that *ica*-independent biofilm development has been described in this microorganism. Our choice regarding *atl*A and *sas*G for *ica*-independent pathway was based on fewer available experimental studies toward their prevalence and correlation with antimicrobial susceptibility of sessile cells, since fibronectin binding factors, FnBPA and FnBPB, are better documented as contributing to the ability of *S. aureus* to adhere to specific surfaces (Greene et al., 1995; Roche et al., 2004).

All isolates harbored *ica*A and *atl*A genes, which are already extensively proved as essential determinants for *S. aureus* biofilm phenotype (Cramton et al., 1999; Biswas et al., 2006; Houston et al., 2011). The expression of *ica*A induces a low enzymatic activity of *N*-acetylglucosaminyltransferase, which is responsible for UDP-*N*-acetylglucosamine synthesis which, in turn, produces PIA (Gerke et al., 1998). On the other hand, the role of SasG in biofilm formation is poorly studied and was first considered as a potential biofilm promoter by Corrigan et al. (2007), a characteristic further demonstrated by Kuroda et al. (2008) with the construction of a *sas*G mutant. So far, SasG was considered as an adhesin to nasal epithelium cells (Roche et al., 2003), but turned out to be an important biofilm protein by facilitating adherence to host tissues in *S. aureus* infections (Kuroda et al., 2008; Geoghegan et al., 2010). Despite SasG being associated with *ica*-independent pathway (Corrigan et al., 2007), we found that isolates with higher OD readings (>0.20) harbored the *sas*G gene. Maybe, this increasing adhesive phenotype operates in a SasG-dependent manner along with PIA, and the *sas*G-mediated aggregation might facilitate increasing cell population for the attachment (Kuroda et al., 2008). Consequently, the lack of *sas*G gene could provide an enhancement to antimicrobial activities in biofilm, which was demonstrated with the rates given by MBEC and MIC values.

This study was able to demonstrate the significant differences between erythromycin, oxacillin, gentamicin, vancomycin, and rifampicin regarding biofilm production intensity by MSSA: the number of cells in a biofilm matrix affects the antimicrobial activity, and this characteristic may also be associated with specific genetic determinants responsible for the expression of biofilm lifestyle. To our knowledge, this was the first study reporting the presence of *sas*G in clinical isolates of *S. aureus* in South America.

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