Article/Artigo

Rifampicin fails to eradicate mature biofilm formed by methicillin-resistant *Staphylococcus aureus*

Rifampicina falha na erradicação de biofilmes maduros formados por *Staphylococcus aureus* resistentes à meticilina

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ABSTRACT

Introduction: Antimicrobial activity on biofilms depends on their molecular size, positive charges, permeability coefficient, and bactericidal activity. Vancomycin is the primary choice for methicillin-resistant *Staphylococcus aureus* (MRSA) infection treatment; rifampicin has interesting antibiofilm properties, but its effectivity remains poorly defined. **Methods:** Rifampicin activity alone and in combination with vancomycin against biofilm-forming MRSA was investigated, using a twofold serial broth microtiter method, biofilm challenge, and bacterial count recovery. **Results:** Minimal inhibitory concentration (MIC) and minimal bactericidal concentration for vancomycin and rifampicin ranged from 0.5 to 1mg/l and 0.008 to 4mg/l, and from 1 to 4mg/l and 0.06 to 32mg/l, respectively. Mature biofilms were submitted to rifampicin and vancomycin exposure, and minimum biofilm eradication concentration ranged from 64 to 32,000 folds and from 32 to 512 folds higher than those for planktonic cells, respectively. Vancomycin (15mg/l) in combination with rifampicin at 6 dilutions higher each isolate MIC did not reach *in vitro* biofilm eradication but showed biofilm inhibitory capacity (1.43 and 0.56$\log_{10}$ CFU/ml reduction for weak and strong biofilm producers, respectively; $p<0.05$). **Conclusions:** In our setting, rifampicin alone failed to effectively kill biofilm-forming MRSA, demonstrating stronger inability to eradicate mature biofilm compared with vancomycin.

Keywords: *Staphylococcus aureus.* Rifampicin. Vancomycin. Biofilm. Resistance.

RESUMO

Introdução: A atividade dos antimicrobianos em biofilmes depende do seu peso molecular, de cargas positivas, coeficiente de permeabilidade e atividade bactericida. Vancomicina é a escolha primária para o tratamento de infecções causadas por *Staphylococcus aureus* resistentes à meticilina (MRSA) e rifampicina possui interessante propriedade antibiofilme, apesar da sua efetividade ainda ser fracamente definida. **Métodos:** Foi investigada a atividade da rifampicina sozinha e em combinação com vancomicina frente à MRSA formadores de biofilme, utilizando o método das microplacas com diluição seriada e recuperação bacteriana em biofilme após exposição antimicrobiana. **Resultados:** Concentração inibitória mínima (MIC) e concentração bactericida mínima (MBC) para vancomicina e rifampicina foi de 0,5-1mg/l e 0,008-4mg/l; 1-4mg/l e 0,06-32mg/l, respectivamente. Biofilmes maduros foram expostos à vancomicina e rifampicina, e a concentração mínima para erradicar o biofilme (MBEC) foi 64-32.000 e 32-512 vezes maior do que para células planctônicas, respectivamente. A combinação de vancomicina (15mg/l) com rifampicina (6-diluições maior do que o MIC de cada isolado) não atingiu erradicação do biofilme *in vitro*, porém apresentou capacidade inibitória do biofilme formado (redução de 1,43 e 0,56$\log_{10}$ UFC/ml para produtores fracos e fortes, respectivamente; $p<0,05$). **Conclusões:** Rifampicina sozinha falhou em efetivamente matar MRSA formadores de biofilme, demonstrando fraca habilidade para erradicação de biofilmes maduros comparado com vancomicina.

Palavras-chaves: *Staphylococcus aureus.* Rifampicina. Vancomicina. Biofilme. Resistência.

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INTRODUCTION

Biofilms provide bacterial cell attachment to an abiotic surface very rapidly, and growth-dependent accumulation form multilayered cell clusters surrounded by a slime-like glycocalix matrix[1]. This matrix confers increased protection against antimicrobials in addition to facilitating adherence to medical devices and cause persistent infections[2]. Antimicrobial activity on biofilms depends on their molecular size, positive charges, permeability coefficient, and bactericidal activity[3], indicating the importance of testing new drugs antibiofilm activity or even trying alternative drug combinations.

Vancomycin is the primary choice for methicillin-resistant *Staphylococcus aureus* (MRSA) infections treatment, although recent studies have demonstrated treatment failures even when the bacteria still is in vitro susceptible to vancomycin[4-7]. This antimicrobial antibiofilm activity already was evaluated and seemed to be highly powerless regarding complete biofilm eradication requirement[8,9].

Rifampicin has putative antibiofilm properties, ability to penetrate staphylococcal biofilm[10], and had demonstrated promising utility as agent for eradicating *S. aureus* biofilm alone[8] or in combination with other drugs especially for device-related infections[11-14]. Nevertheless, its effectivity remains poorly defined because few and limited supporting human studies have been performed[11,14]. Moreover, recently, in vitro studies have demonstrated antagonistic rifampicin effects in experimental foreign body infection models[15].

To evaluate antimicrobial behavior in biofilm, rifampicin and vancomycin activities alone and in combination against device-related MRSA were investigated.

METHODS

Bacterial isolates

Five known biofilm-producing MRSA (H142SA, H290SA, H369SA, H403SA, and H410SA) previously obtained from five different patients with

device-related bloodstream infections at Complexo Hospitalar Santa Casa de Misericordia de Porto Alegre (Porto Alegre, Brazil) were evaluated. These isolates were selected from positive blood cultures and previously assessed for biofilm-producing ability, *mec*A and SCC*mec* typing, and antimicrobial susceptibility pattern **(Table 1)**[16].

TABLE 1 - Methicillin-resistant *Staphylococcus aureus* isolates characteristics.

Isolate	SCC*mec*	Biofilm category	Susceptibility pattern*
H142SA	I	strong	Dox, Ery, Cli, Sxt, Lzd, Syn
H290SA	III	weak	Dox, Lzd, Syn
H369SA	III	strong	Lzd, Syn
H403SA	I	moderate	Dox, Sxt, Lzd, Syn
H410SA	IVb	weak	Dox, Sxt, Lzd, Syn

SCC*mec*: staphylococcal cassette chromosome *mec*; * **Antimicrobials: Dox**: doxycycline; **Ery:** erythromycin; **Cli:** clindamycin; **Sxt:** sulfamethoxazol-trimethoprim; **Lzd:** linezolid; **Syn:** quinupristin-dalfopristin. All MRSAs were resistant to gentamicin and ciprofloxacin.

Minimum inhibitory concentration and MBC testing

Conventional minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of vancomycin and rifampicin were determinate by twofold serial broth microdilution according to CLSI (2009) guidelines[17]. *Staphylococcus aureus* ATCC 29213 was tested as quality control. Vancomycin and rifampicin analytical powder was provided by Sigma-Aldrich (St. Louis, MO, USA).

Biofilm susceptibility tests

Minimal inhibitory concentration in biofilm (MIC^{ADH}) and minimum biofilm eradication concentration (MBEC) experiments were performed as described elsewhere[8], with a serial twofold dilution of each antimicrobial in cation-adjusted Mueller-Hinton broth. Minimum inhibitory concentration^{ADH} was defined as the minimal antimicrobial concentration at which there was no observable bacterial growth in the wells containing adherent microcolonies, in other words, the minimal concentration that inhibits the bacterial growth. Minimum biofilm eradication concentration was defined as the minimal antimicrobial concentration at which bacteria fail to regrow after antimicrobial exposure, that is, the minimal concentration required to eradicate the biofilm. All determinations were performed in duplicate. Rifampicin MBEC values also were determined using an alternative method[18], to compare and confirm the results. It was also performed in duplicate.

Biofilm challenge and recovery

Standard vancomycin concentration corresponding to clinical pharmacokinetic trough concentration goal of 15mg/l[19], rifampicin at 6-dilution higher each microorganism MIC, and vancomycin 15mg/l in combination with rifampicin 6-dilution higher each microorganism MIC were used in biofilm challenge according to Raad et al.[20] with some modifications. Briefly, biofilms formed on the MRSA microtiter plates' bottom were rinsed twice with sterile saline and submitted to antimicrobial exposure. Challenged biofilms were washed twice in sterile saline and placed with fresh trypticase soy broth (TSB), and the remaining biofilm was mechanically disrupted. Bacterial count recovery was determined by 1-µl culture on trypticase soy agar (upper detection limit 6$\log_{10}$ colony-forming units per mililiter (CFU/ml)), in quadruplicate. Bactericidal activity was defined as a 3$\log_{10}$ CFU/ml) or greater reduction (99.9% kill) from the untreated biofilms[21]. Only rifampicin-susceptible isolates were tested and organized into weak (H290SA and H410SA) and strong/moderate (H142SA and H403SA) biofilm producers.

Statistical analysis

The difference between positive control (without antimicrobial exposure) and each isolate after antimicrobial exposure was characterized as Δlog reduction, in $\log_{10}$ CFU/ml. The variables investigated were the antimicrobial tested (vancomycin, rifampicin or the association of both) and intensity of biofilm production (weak or strong), which were analyzed by applying two-tailed independent samples *t* Student test with significant p value of 0.05 or lower. All statistical tests were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

All isolates were susceptible to vancomycin by MIC determination. Only H142SA was the one not considered multiresistant but demonstrated strong biofilm formation ability and SCC*mec* type I.

Vancomycin MBC was constantly one dilution higher than MIC values for all tested isolates, and MBEC ranged from two to six dilutions higher than MIC^{ADH} values. Only H410SA on biofilm remained within vancomycin susceptibility breakpoint. However, its MBEC was six dilutions higher than MIC^{ADH} **(Table 2)**.

High rifampicin MBEC/MIC ratio and MBEC measurements six to fifteen dilutions higher than MIC were observed. Strong biofilm producers presented higher MBEC values than weak biofilm producers, same with MIC^{ADH} values. Both methods used for rifampicin MBEC testing showed very similar results **(Table 2)**.

Rifampicin-susceptible isolates CFU/ml counting was performed. Rifampicin at 0.5mg/l and vancomycin at 15mg/l did not achieve bactericidal activity at 24h, same with combination of both drugs. Log_{10} CFU/ml reduction was significantly different between weak and strong biofilm producers ($p < 0.05$) and among all antimicrobials tested ($p < 0.05$) **(Figure 1)**.

TABLE 2 - Rifampicin and vancomycin susceptibility results for planktonic and sessile cells.

Susceptibility results[a]	sH142SA	wH290SA	sH369SA	mH403SA	wH410SA
Planktonic cells					
rifampicin					
MIC (mg/l)	0.008	0.008	4	0.008	0.008
MBC (mg/l)	0.06	0.06	32	0.125	0.06
vancomycin					
MIC (mg/l)	1	2	0.5	1	1
MBC (mg/l)	2	4	1	2	2
Sessile cells					
rifampicin					
MIC^{ADH} (mg/l)	64	32	64	32	16
MBEC (mg/l)[b]	256	64	256	64	64
MBEC (mg/l)[c]	128	64	256	64	64
MBEC/MIC ratio	32,000	8,000	64	8,000	8,000
vancomycin					
MIC^{ADH} (mg/l)	8	8	64	8	2
MBEC (mg/l)	64	64	256	128	128
MBEC/MIC ratio	64	32	512	128	128

Lowercase letter before each isolate means the biofilm category (strong, moderate, and weak producer). [a] rifampicin (≤1mg/l); vancomycin (≤2mg/l). CLSI range susceptibility. [b] MBEC assay according Cafiso et al; [c] MBEC assay according Antunes et al. **MIC**: minimal inhibitory concentration; **MBC**: minimal bactericidal concentration; **MIC^{ADH}**: minimal inhibitory concentration in biofilm; **MBEC**: minimum biofilm eradication concentration.

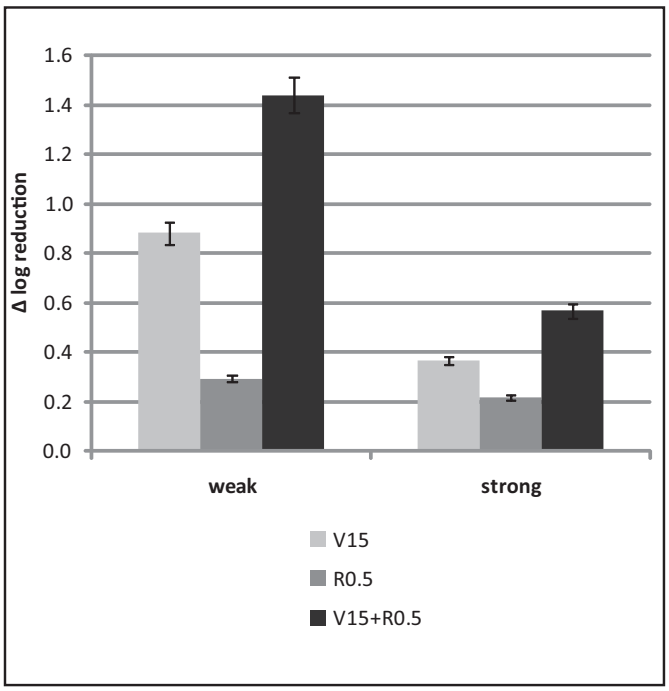

FIGURE 1 - Effect of vancomycin (V15), rifampicin (R0.5), and both drugs in combination (V15+R0.5) against weak and strong MRSA biofilm producers after 24-h exposure.

Δ log reduction: difference between positive control (without exposure: 6 $\log_{10}$ CFU/ml) and after exposure in $\log_{10}$ CFU/ml. Error bars represent standard deviation.

DISCUSSION

Device-related infections have been associated with bacteria embedded in biofilm[11,22,23], and rifampicin could be used as additional therapy in foreign body-related infections due to MRSA[24]. Otherwise, in our setting, vancomycin is preferable as antimicrobial coverage, and rifampicin is unusually prescribed. Because studies have demonstrated that rifampicin in combination with other drugs might be more effective[12,13] despite contradictory results[15], we decided to investigate rifampicin activity alone and in combination with vancomycin against biofilm-forming MRSA.

Distinct research groups have investigated anti-Gram-positive drug activity, alone or in combination with other agents, against biofilm-forming bacteria. However, not all studies are comparable in terms of results concordance[8,12,20,25-28]. In this study, vancomycin was not able to inhibit adherent cells or eradicate mature biofilms at the same concentration necessary for killing planktonic cells. Likewise, MIC^{ADH} and MBEC values were widely distant from each other; biofilm-eradicating concentrations varied from 8- to 64-fold higher than biofilm-inhibiting concentrations. Vancomycin susceptibility against biofilm-forming staphylococci was previously studied in Brazil[9] and showed alarming results—as also demonstrated in this study—because this drug is the primary choice for antimicrobial and empirical treatment.

Unlike other studies[8,12,13], we demonstrated that rifampicin alone is worse than vancomycin for inhibiting staphylococci embedded in biofilm. On the other hand, rifampicin in combination with vancomycin at 15mg/l inhibited bacterial grown in biofilm and, therefore, improved vancomycin activity, because of rifampicin's better biofilm penetration[10,20]. Rifampicin associated with other antimicrobials, for example, gentamicin and clindamycin, may be a better strategy and also more effective than rifampicin alone[29], but all MRSA in our study were resistant to both drugs, and this combination would not be appropriate in this case.

Bacterial growth inhibition occurred with rifampicin in combination with vancomycin, but absence of biofilm eradication may contribute to persistence of biofilm-forming bacteria in the human body. Further and more specific studies in our setting regarding rifampicin activity in biofilm are necessary to fully understand its place in biofilm-related MRSA infection treatment, but this antimicrobial could be considered an interesting candidate for enhancer of antistaphylococcal activity combined with more bactericidal agents.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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